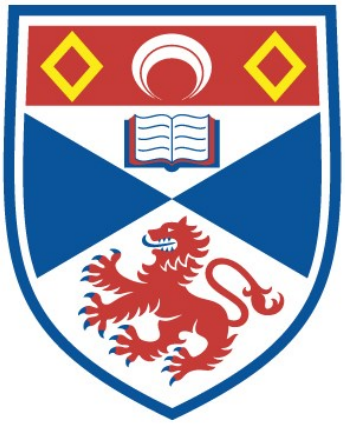
THE ASSOCIATION OF HIGHER FUNGI AND SAND-DUNE GRASSES

Ruby Ione McKay

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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THE ASSOCIATION OF HIGHER FUNGI AND SAND-DUNE GRASSES.

by

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A thesis submitted to the University of St. Andrews for the degree of Doctor of Philosophy.

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D E C L A R A T I O N.

I hereby declare that the following Thesis is based on a record of work done by me, that the Thesis is my own composition, and that it has not previously been presented for a Higher Degree.

The research was carried out in the Department of Botany at St. Salvator's College of the University of St. Andrews under the direction of Professor J. A. Macdonald.

C E R T I F I C A T E.

I certify that Ruby I. McKay has spent nine terms of research work under my direction and that she has fulfilled the conditions of Ordinance No. 16 (St. Andrews), and that she is qualified to submit the accompanying Thesis in application for the degree of Doctor of Philosophy.

C A R E E R.

In 1959, I was awarded a Jamaica Government Teacher's Scholarship tenable at the University of the West Indies, Kingston, Jamaica, from which I graduated in July, 1963 with a first class honours Bachelor of Science (London) degree in Botany and Zoology.

I taught Botany and Biology in St. Andrews High School for Girls in Kingston, Jamaica for two years.

I was then awarded the Sir James Irvine Memorial Scholarship, tenable at the University of St. Andrews, Scotland.

I matriculated at the University of St. Andrews in October, 1965 and was admitted as a Research Student under Ordinances 16 and 61.

During the tenure of the Scholarship I undertook the research work presented here for the degree of Ph. D.

A C K N O W L E D G E M E N T S.

I wish to express my thanks to Professor J. A. Macdonald, my supervisor who suggested the problem, for his advice and interest during the course of this work.

I am also indebted tothe Statistics Department of thb University of St. Andrews for statistically analysing the data in Sections 5a and 5d of this thesis.

I would like to take the opportunity to express my gratitude also to the University of the West Indies for awarding me the Sir James Irvine Memorial Scholarship and to Sir Harold Mitchell Bt., M.A., Hon. LL.D., K. St. J., who provided the funds, thus making it possible for me to carry out the work presented here.

C O N T E N T S

S E C T I O N 1

INTRODUCTION

During recent years the biological and chemical composition of sand-dunes has been the subject of fairly extensive investigations. The contribution to the knowledge of the micro-organisms in that ecological niche is mainly based upon the work of Moreau (1941, 1942), Webley, Eastwood & Gimingham (1952), Brown (1958), Nicolson (1959, 1960), Pugh (1962), Pugh, Blakesman, Morgan-Jones and Eggins (1963) and Hassouna and Wareing (1965). That the array of lower fungi in sand-dunes is very wide was shown by Moreau (1941, 42), Pugh (1962) and Pugh et al (1963). Soil fungi of some British sand-dunes in relation to soil type and colonization by higher plants have been studied by Webley et al (1952) and Brown (1958). Hassouna and Wareing (1965) contributed by discussing the possible role of rhizosphere bacteria in the nutrition of Ammophila arenaria L. while Nicolson's (1959,60) contribution was a study of the vesicular-arbuscular type of mycorrhiza in grasses confined to this area.

Dobbs and Gash (1965) have reported the presence of microbial and residual mycostasis in soils with low nutrient conte nt e .g. sand-dunes. The nutrient status of the dunes has been investigated by Webley et al (1952), Willis and Yemm (1961) and Oliver (1967) and its effects on the vegetation by Willis (1963, 1965). The results of these studies have been fairly consistent and can be briefly summarised as follows:- some of the dunes are acidic while others are alkaline. On the whole, the mineral content is very low except for sodium, carbonates and chlorides. This higher content is to be expected since

the area receives sea sprays which contain appreciable quantities of these ions. Potassium, nitrogen and phosphorus have been reported to be extremely low in quantity in the bare sand as well as in the colonized areas of the fore dunes.

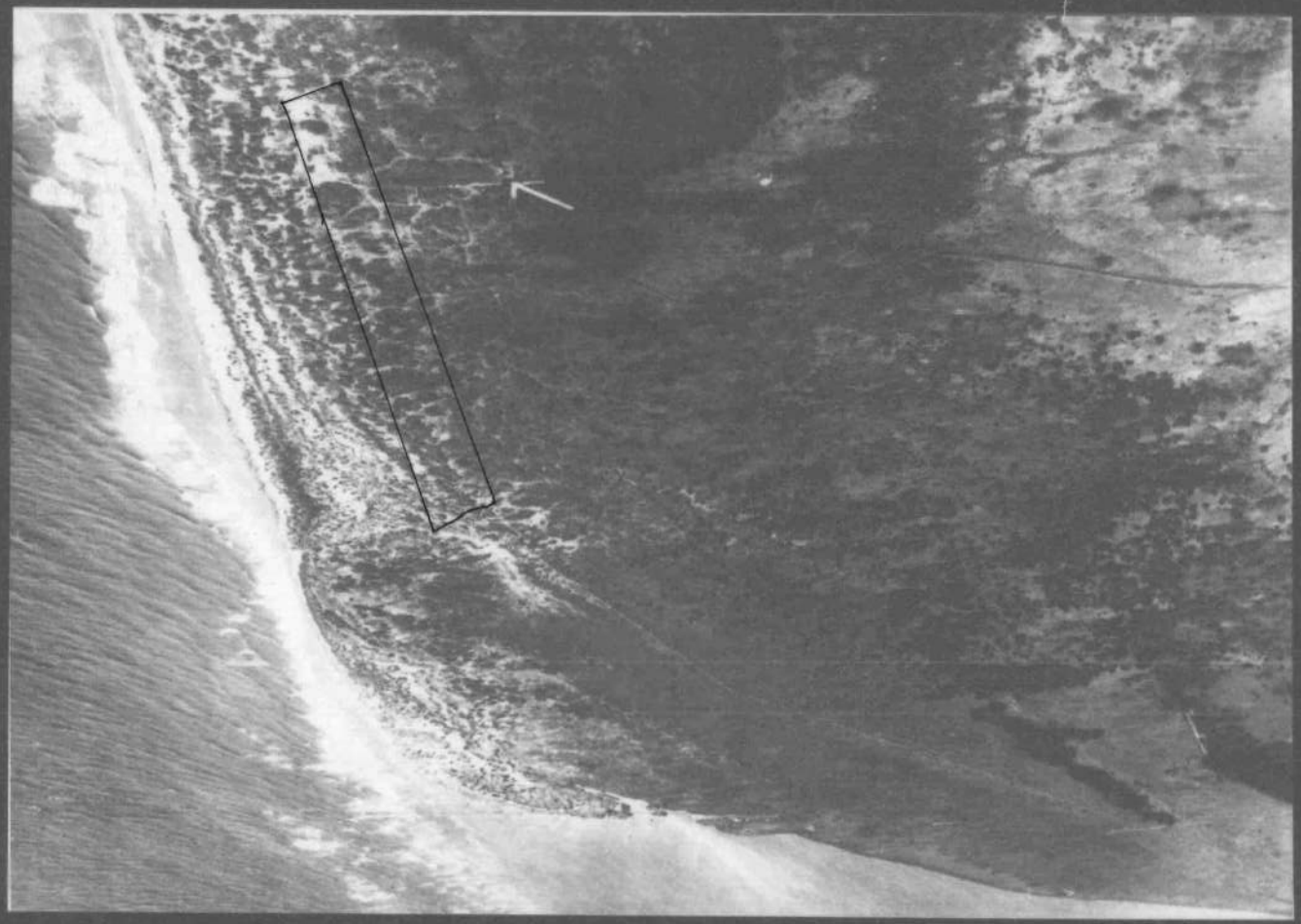
Although fruit bodies of the higher fungi have been observed on the sand-dunes since the last century, very little is known about their nutrition. The purpose of this exercise is, therefore, an attempt to find out whether there is a nutritional relationship existing between the higher fungi and the grasses. Certainly, they show specialization by growing only on the dunes where particular grasses predominate, and the regularity with which they fruit each year suggests that there is a link between these two groups of plants.

This work was carried out on the fore dunes (between 20 and 40 feet above high water mark) at the Nature Reserve in Tentsmuir, Fife (Plate 1, Grid ref. 56/5026 OS map of Great Britain). In this region, coastal accretion is proceeding more rapidly than in almost any other part of Britain. This rapid gaining of ground from the sea affords exceptional opportunities for the study of the colonization by higher plants and fungi. The three dominant species of higher plants which colonize the dunes are Agropyron junceiforme A. & D. Love, Elymus arenarius L. and Ammophila arenaria L.. A. junceiforme is more abundant and A. arenaria less abundant towards the high water mark. The reverse is true as the dune becomes more fixed. E. arenarius occupies an intermediate position.

The fruit bodies of three Agarics and one Discomycete occur in high frequency on the dunes among the grasses (Plates 2-5). There is a good deal of confusion concerning

PLATE 1

An aerial view of the sand - dunes at Tentsmuir. The area where the investigation was carried out is outlined.



the nomenclature of these fungi but after referring to Saccardo (1888 and 1889), Lange (1936-40), Metrod (1941, 1942), Andersson (1950), Dennis (1960), Orton (1960), Dennis, Orton and Hora (1960), Singer (1962) and Watling (personal communication) the following names have been ascribed to the regularly occurring sand-dune fungi, (i) Conocybe dunensis, Wallace 1960.

(ii) Melanoleuca grammopodia (Fries ex Bulliard) Pat. 1900. Many accounts of sand-dune fungi including that of Wallace (1954), refer to this fungus as Tricholoma melaleucum (Pers. ex Fr. 1921) Kummer 1871, but the amyloid warts of the spores and harpoon-shaped cystidia keep it in the genus Melanoleuca (Orton, 1960 and Singer, 1962).

(iii) Psathyrella ammophila (Durieu et Léville) Orton 1960. This fungus has previously been recorded, for example, by Andersson (1950) and Wallace (1954) as an obligate sand-dune fungus under the name of Psilocybe ammophila (Durieu et Léville, 1868) Gillet 1874, but because of its distinct cellular epicutis it has been given the newly combined name by Orton (1960).

(iv) Peziza ammophila (Durieu et Montagne) Cooke. This Discomycete has been called Geopyxis ammophila in many papers which deal with sand-dune fungi e.g. Wallace (1954) because of its stalk-like base but the iodine reaction of the asci is a more important diagnostic feature (Dennis, 1960).

The conditions of the mobile dunes seem at first sight to be most unfavourable for the nutrition, growth and fruiting of the larger fungi. Wallace (1954) reported that the mycelia of these fungi grow in close association with the material from which they get their nourishment. He stated that "they grow among decaying plant remains, in the

PLATE 2

C. dunensis growing in association with *A. junceiforme* and *E. arenarius* on the sand-dune in Tentsmuir.

PLATE 3

M. grammopodia growing close to a tuft of *A. arenaria* - hence the distorted fruit body.

PLATE 4

Psathyrella ammophila and *E. arenarius* on the fore - dune in Tentsmuir.

PLATE 5

Peziza ammophila and *E. arenarius*.

humus layer underground, in dead wood, in dung or in mycorrhizal partnership with the living roots of the higher plants". These observations are very generalized and the current investigation was designed to find out if the fungi were in fact more specific in their nutritional requirements.

The work comprised the isolation of the fungi in culture, a study of some of their growth requirements, and their ability to produce extracellular enzymes which would make them successful competitors in the field. An attempt has also been made to get them to form synthetic mycorrhiza and consideration has been given to the differences between cultural conditions and the natural environment of the fungi and the grasses.

SECTION 2

MATERIALS AND METHODS

2a Materials

All the materials used in this investigation were collected from the foredunes of the Nature Reserve at Tentsmuir in Fife.

The caryopses of the grasses A. arenaria, A. junceiforme and E. arenarius were chosen in November, 1966 when they appeared to be dry. In the laboratory they were husked and stored in closed bottles at room temperature until required.

For the experiments requiring root extracts, the roots were collected in August, 1967 and those required for isolation of fungi were collected in September, 1966 and May, 1967.

In the experiments 5a and 5d where sand was used, it was taken from a depth of 6-12 inches from about 12 areas at random where the grasses and fungi flourished, and then thoroughly mixed before use.

The fruit bodies of the fungi C. dunensis, M. grammopodia, Psathyrella ammophila and Peziza ammophila were collected at or near the beginning of the ir growing seasons (Table 1) and isolations made as described in Section 2b(iv).

Table 1

Fruiting Season

Fungus	1966	1967
Psathyrella ammophila	1st wk Aug.- 4th wk Nov.	2nd wk July- 3rd wk Nov.
Peziza ammophila	1st wk Aug.- 4th wk Nov.	2nd wk July- 3rd wk Nov.
M. grammopodia	2nd wk Aug.- 1st wk Dec.	3rd wk Sept.- 1st wk Dec.
C. dunensis	1st wk Sept.- 1st wk Dec.	3rd wk Sept.- 1st wk Dec.

Unless otherwise stated the reagents were supplied by British Drug House and were of Analar grade.

2b General Methods

(i) Sterilization

(a) Glassware

Petri plates were sterilized with dry heat at 180°C for two hours. Unless otherwise stated, the Erlenmeyer flasks were acid washed, rinsed in distilled water, plugged with non-absorbent cotton wool and sterilized by autoclaving at 15 lbs. pressure per square inch for fifteen minutes.

(b) Culture media

All the culture media used were sterilized by autoclaving at 15 lbs. pressure per square inch for fifteen minutes, except the nitrogen sources. The nitrogen compounds were dissolved in distilled water and steam sterilized for half an hour on each of three successive days. When glucose was one of the constituents of any of the media, it was sterilized separately.

(c) Caryopses

After the caryopses were husked by hand, they were soaked in water for 1-2 hours and all traces of lemma and palea were removed with a pair of forceps and/or a scalpel. This was to allow the disinfectant to reach the entire surface of the grains. Care was taken not to damage the embryos during the operation.

The caryopses were then soaked overnight in sterilized 0.1% difco agar in distilled water which acted as a wetting agent (Chesters, personal communication 1966) and then surface sterilized by soaking in a saturated solution of sodium hypochlorite which contained 10-14% weight/volume available chlorine for fifteen minutes. This treatment proved too drastic for E. arenarius and A. juncciforme resulting in very poor germination. The sodium hypochlorite was then diluted to 50% and 10%. The caryopses were again soaked for fifteen minutes and then washed in six changes of sterilized distilled water to remove all traces of the sterilizing agent.

(d) Fructification for Isolation

Small portions of gills of young basidiocarps of C. dunensis, M. grammopodia and Psathyrella ammophila and sections of the hymenium of the apothecium of Peziza ammophila were aseptically excised and surface sterilized with 5% sodium hypochlorite for one minute. The results were similar when 1.5% sodium hypochlorite was used for fifteen minutes.

(e) Roots for Isolation

When roots collected from Tentsmuir were seen by microscopic examination to be associated with Basidiomycetes, they were cut in pieces 5mm long, surface sterilized with 5% sodium hypochlorite for

fifteen minutes and then washed in six changes of sterilized distilled water.

(f) Earthenware Pots and Sand

The new 5" earthenware flower pots which were used in the experiment to be described in Section 5a, were covered with aluminium foil to prevent them from being flooded during sterilization. They were then sterilized by autoclaving at 15 lbs. pressure per square inch for three hours.

The soil used in Series 1 of the same experiment was sand which was collected from Tentsmuir (See the section on Materials). It was passed through a 2mm mesh sieve and mixed with vermiculite in the ratio of three parts sand to one part vermiculite and then autoclaved at 15 lbs. pressure for four hours. In addition to autoclaving, McArdle (1932) heated his sand at 105°C for six hours. It was found that four hours in the autoclave was sufficient to kill all micro-organisms and so heating in the oven was omitted.

The method of sterilization used for Series 2 of the same experiment was different. In this case, the sand was not sieved and no vermiculite was added. The plant parts found naturally in the sand were incorporated after be ing out in small pieces. The sand was then steam sterilized for one hour on each of three successive days. It was found that half an hour was inadequate as fungi and bacteria grew from the sand and debris when plated on malt extract agar.

2b (ii) Examination of Roots for Infection

Roots from plants in Tentsmuir

In September, 1966 and May, 1967 the underground parts of A. arenaria, A. junceiforme and E. arenarius were collected from Tentsmuir. Holes, about 12 inches deep, were made in the foredune and the plants with as much of their root system as possible, were removed and taken to the laboratory. The excess sand was removed by washing and the roots which occupied the top six inches in the sand on the dunes were cut off, washed, placed in distilled water and aerated while awaiting examination. These and subsequent steps were hastened to prevent changes in the cells e.g. atrophy.

Free-hand longitudinal sections were made and stained with cotton blue in lactophenol and examined microscopically in lactophenol. Between three and six sections were made from each root according to the length of the particular root. The presence of Basidiomycetes was recorded when hyphae with clamp-connections were observed on or within the roots.

Roots of plants grown in the greenhouse

These plants were carefully removed from the pots and gentle shaking removed the bulk of the sand. The root systems were then detached from the aerial parts and washed gently to remove as much of the sand as possible without removing ectotrophic mycelium, if present. The primary roots were carefully separated from one another for examination purposes.

The individual roots were examined under a binocular microscope (X 10) but, except in the case of E 1, no infection was observed at this magnification.

Tangential longitudinal sections were made by hand, stained with cotton blue in lactophenol and examined in lactophenol at X 100 and X 400 magnifications. An average of five sections were made from the first 10cm of the roots when all that was required was to establish the presence or absence of the fungus. When infection was observed in roots from one plant, the extent of infection was studied on two of those roots chosen at random. The roots were very long, reaching a length of just over 200cm. They were cut in 10cm lengths and one section made from each length until the fungus ceased to be present.

If the fungus was observed in and/or on the roots, the infected parts were fixed in formalin-acetic acid-alcohol fixative and embedded in Parowax following Johanssen's technique (Johanssen, 1940). Transverse and longitudinal sections were cut 10-15 μ in thickness. Cotton blue in lactophenol was used, as this stain proved superior to magdala red and light green; magdala red and fast green; picro aniline blue; saffranin and fast green. These sections were used for more detailed study and for photography.

2b (iii) Fungal Isolations from Roots

Isolations were attempted within 36 hours of removing the roots from the sand. The roots were surface sterilized as shown in the Section 2b(i)(e) in Methods.

Longitudinal sections of the cortex were then made with a surface sterilized blade and 4-5 of them were put in each petri plate and gently pushed into the isolation medium. The sections ranged from 1-3mm in length.

Two different media were used:-

(a) Martin's medium (Johnson et al, 1960)

Agar	20.0 grams
Potassium dihydrogen phosphate	1.0 "
$MgSO_4.7H_2O$	0.5 "
Peptone	5.0 "
Dextrose	10.0 "
Distilled water	1000 Ml
Rose bengal	35 mg
Streptomycin	30 " (Dista Product Ltd. Liverpool)

The rose bengal was dissolved in distilled water and added to the medium before sterilization. The streptomycin was made up in 20% alcohol and added to the medium after sterilization.

(b) Malt extract agar + dextrose (Raper & Thom, 1949)

Agar	25.0 grams	(Difco bacto agar)
Malt extract	20.0 "	(Boots)
Dextrose	20.0 "	
Peptone	1.0 "	
Distilled water	1000 ml	

The plates were incubated at 23°C and examined daily.

When the sections were contaminated by bacteria, they were washed in streptomycin (30 mg/1000 ml) and re-plated on new media. Sometimes this had to be repeated two or three times before bacteria were completely eliminated. On many occasions this resulted in the death of the emerging hyphae. Daily microscopic observations enabled the Basidiomycetes to be isolated from other fungi by subculturing.

2b (iv) Fungal Isolation from Fructifications

C. dunensis was isolated in November, 1965 while M. grammopodia, Psathyrella ammophila and Peziza ammophila were isolated in August, 1966.

The fruit bodies were removed carefully from the sand-dunes and placed in sterilized containers. They were taken to the laboratory where isolations were made within twelve hours. The best results were obtained when isolations were made before a time lapse of two hours.

In all cases it was found that isola tions were more successful when attempted early in the growing season. It would appear that as the growing season progressed the number of Phycomycetes and Bacteria which grew from the inoculants increased and the slow growing Basidiomycetes were quickly overgrown by the contaminants.

With the exception of M. grammopodia, the pilei are very thin and gills were used for isolation. Uncontaminated growth was practically impossible without surface sterilization and when sterilization was enough to prevent growth of the contaminants, the inoculants also failed to grow. The same applied to the hymenium of Peziza ammophila.

The inoculants were sterilized as shown previously under "Sterilization". By means of a sterilized loop, they were transferred from the sterilizing agent to petri plates containing malt extract agar. This isolation medium had the following constituents:-

Malt extract	37 grams
Difco agar	20 "
Distilled water	1000 ml

It was sterilized by the technique shown under "Sterilization".

The plates were incubated at 23°C and were examined periodically. Growth was observed after about one week and subcultures were made then, or as soon as the hyphae had grown 2-5mm, depending on the presence or absence of contaminants.

In cases of Peziza ammophila, the spores germinated in 2-3 days and subcultures were made on the 4th or 5th day.

2b (v) Growth of Seedlings

The caryopses were collected in November, 1966 and germinated between January and April, 1967.

Seedlings were obtained under two different conditions. Those which were used in the inoculation experiment to be described in Section 5a, were grown on filter paper in crystallizing dishes, while those used in Experiment 5d - production of root exudates and their effect on the growth of isolates - were grown in sterilized sand in one litre beakers.

Four 9cm Whatman No. 4 filter papers were torn in small pieces and placed in each crystallizing dish. Thirty ml distilled water were added and two whole 9cm filter paper discs were put on top of the torn ones. This was to increase the amount of water in the dishes without the risk of drowning the "seeds". The dishes were then covered with a lid and sterilized as described under Section 2b(i)(b).

The caryopses were sterilized using the technique described under "Sterilization" and aseptically transferred to the dishes. They were incubated at 23°C in the

dark except when light was admitted during examination. As soon as they had germinated they were transferred to a window sill at room temperature.

For the root exudate experiment, the caryopses were surface sterilized as described in Section 2b(i)(c) and were put to germinate in one litre beakers $\frac{2}{3}$ full of sterilized sand. The beakers were then put under large bell jars in a warm dark room until germination commenced after which time they were given artificial light for nine hours daily.

The side of the bell jar and the sheet of glass on which it rested were swabbed with 70% alcohol. The bell jars were used to prevent excessive evaporation. It was hoped that this would also keep out air borne fungal and bacterial spores and so keep the seedlings as sterile as possible.

SECTION 3

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF THE ISOLATES

A. Conocybe dunensis

This is a relatively slow growing fungus. At 23°C the average growth rate on 2% malt extract agar is 3.7mm per week. The mycelium appears white with very little aerial hyphae. On agar plates the fungal colony is often zoned. Clamp connections are present. Sometimes straight and narrow hyphae may be present but usually they become bulbous just behind the growing point - a bolbitiaceous characteristic (Plates 6 and 6a).

B. Melanoleuca grammopodia

This is also a slow growing fungus increasing at the rate of 3.5mm per week on 2% malt extract agar at 23°C. Aerial hyphae are absent. It also has large hyphal protuberances (Plates 7 and 7a) and clamp connections are absent. It causes the agar in its vicinity to become blackish, perhaps because of excretion of substances in the agar.

C. Psathyrella ammophila

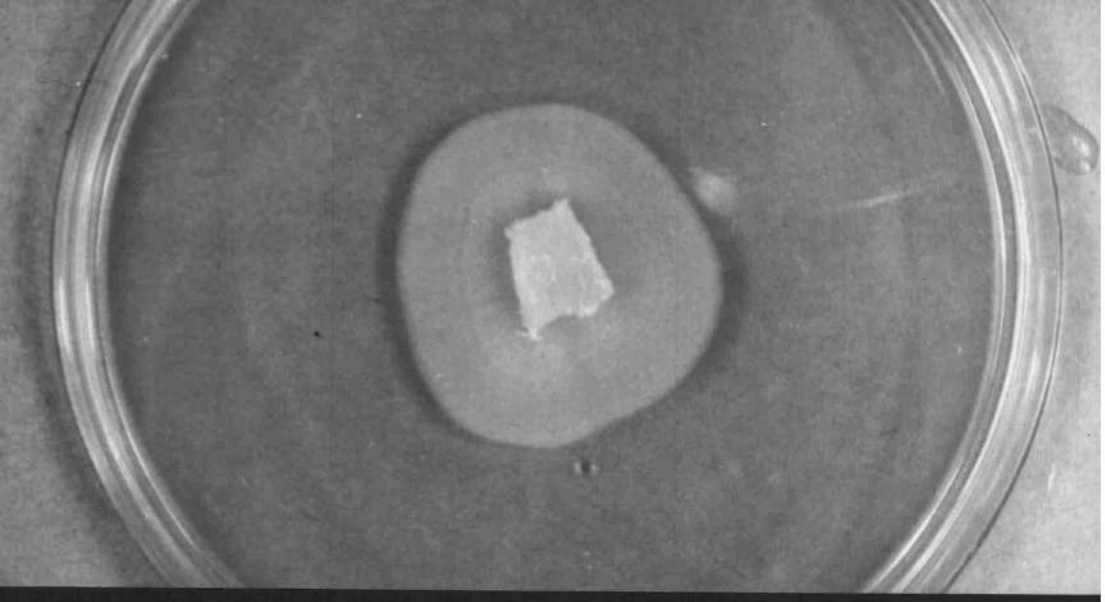
This grows relatively fast and under cultural conditions similar to those of C. dunensis, it grows at an average of 10.4mm per week. Aerial hyphae are short and dense; at first the hyphae are white but they become light brown with age. All hyphae are branched and straight and richly provided with clamp connections (Plates 8 and 8a).

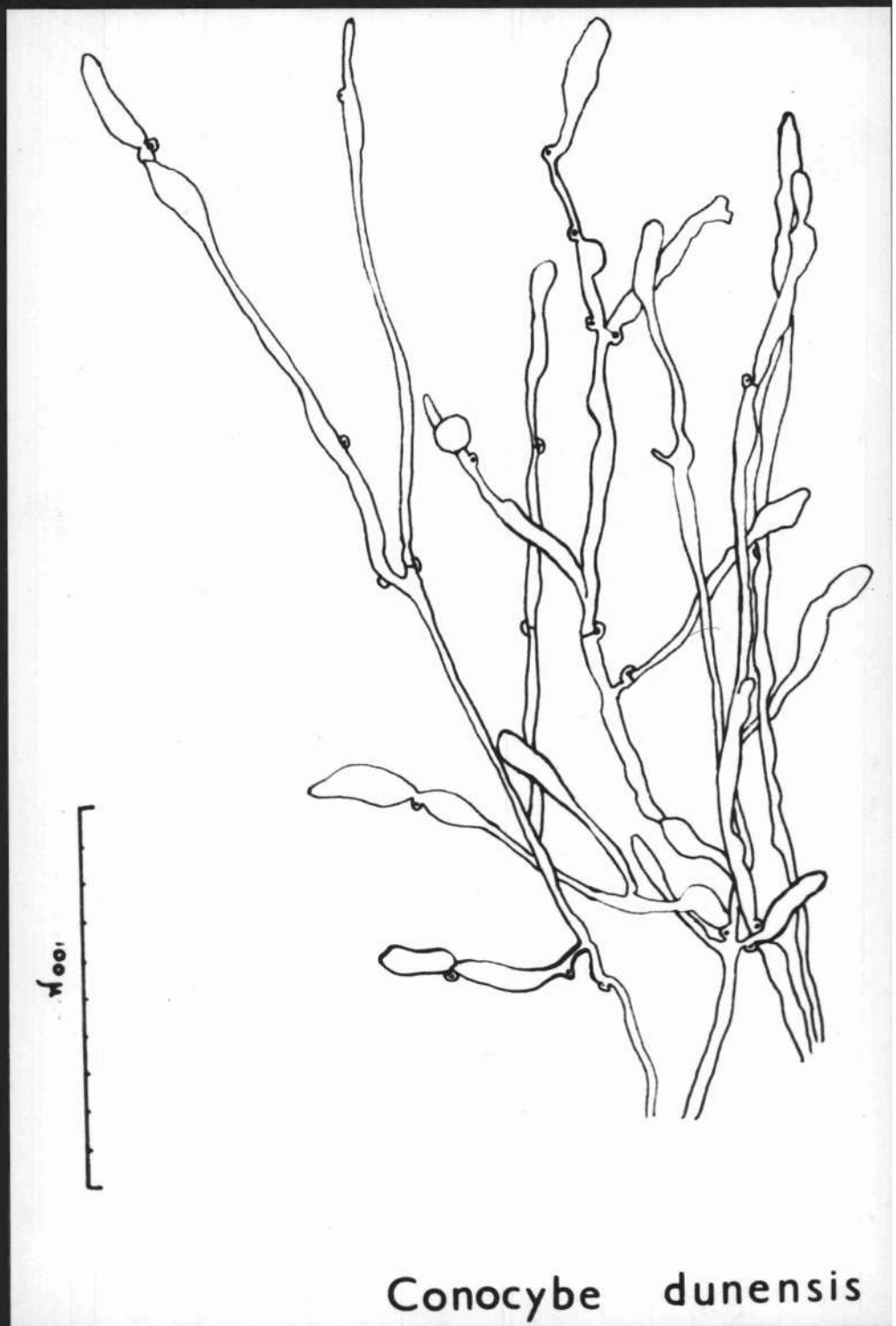
PLATE 6

A colony of *C. dunensis* growing on malt extract agar.

PLATE 6a

Camera lucida drawing of hyphal tips of *C. dunensis* taken from the colony shown in Plate 6.





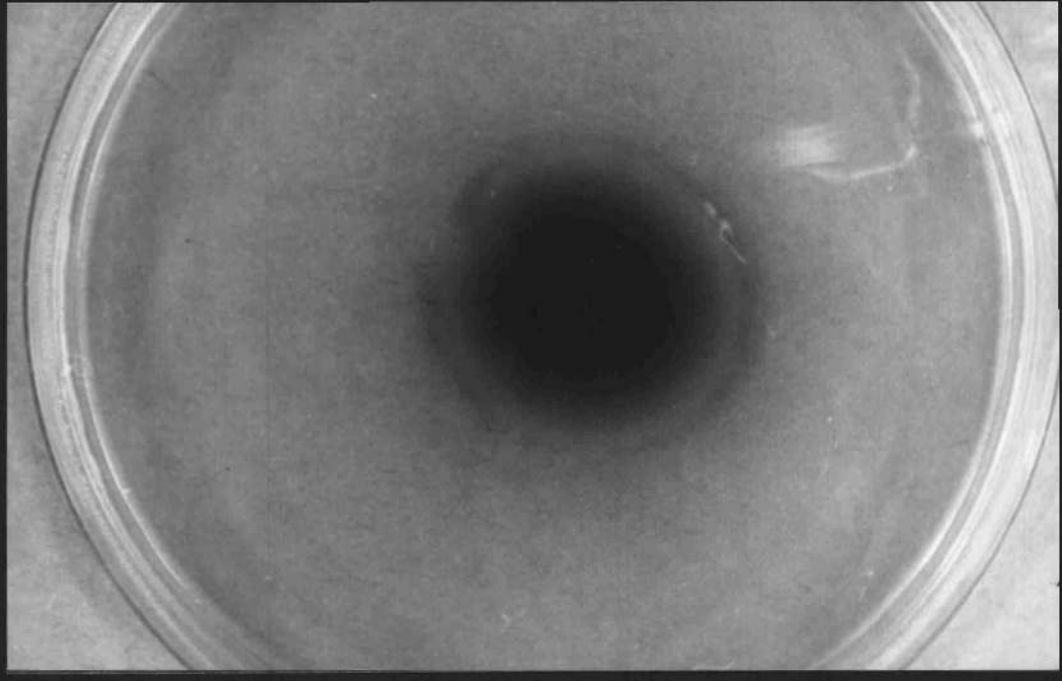
Conocybe dunensis

PLATE 7

A colony of M. grammopodia growing on malt extract agar. The characteristic discoloration of the agar is clearly demonstrated.

PLATE 7a (i)

Camera lucida drawing of hyphae from the advancing edge of the colony shown in Plate 7.



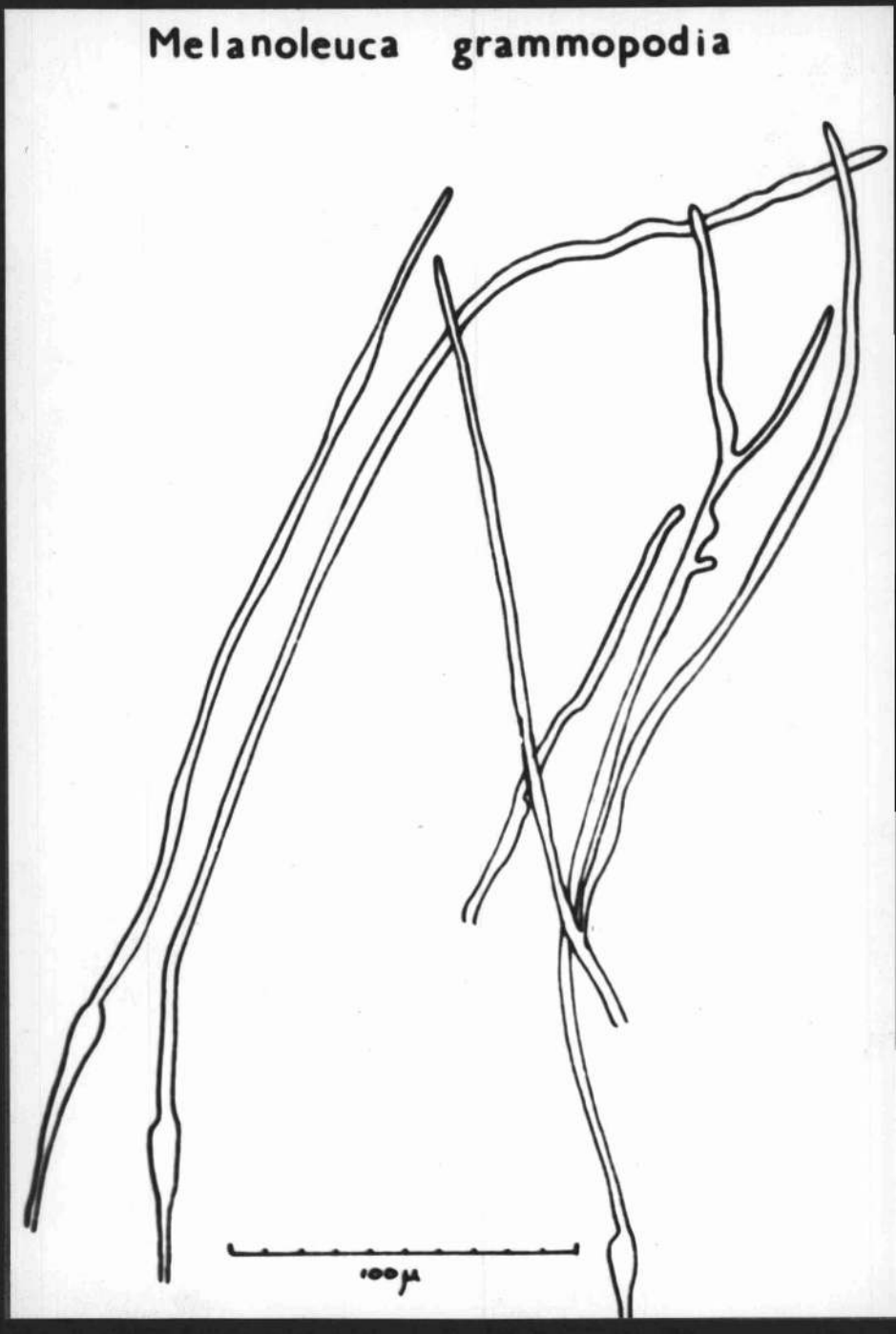

Melanoleuca grammopodia
100μ

PLATE 7a(ii)

Camera lucida drawing of hyphae from the older regions of the colony of *M. grammopodia* in Plate 7 illustrating the bulbous nature of the hyphae.

Melanoleuca grammopodia

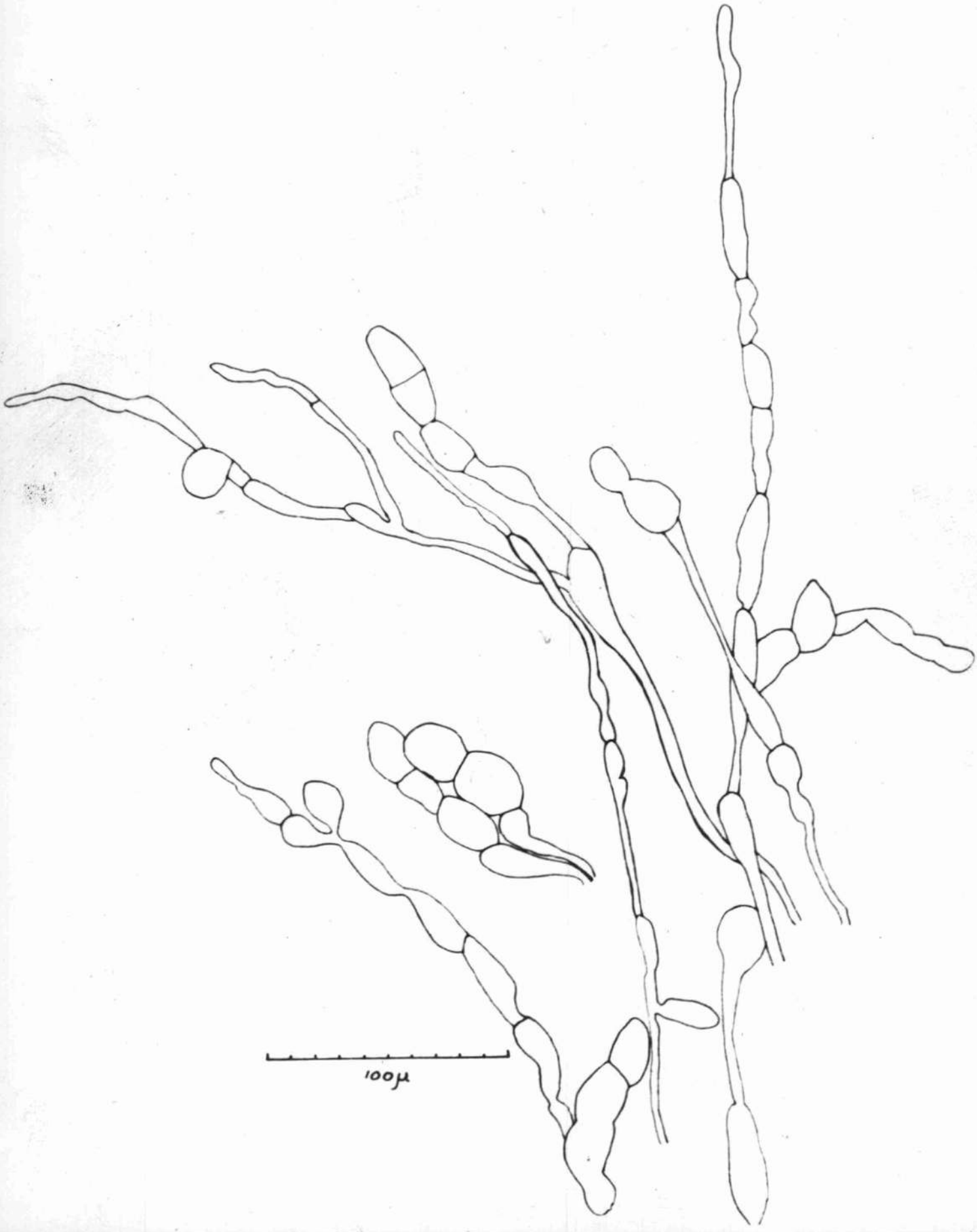
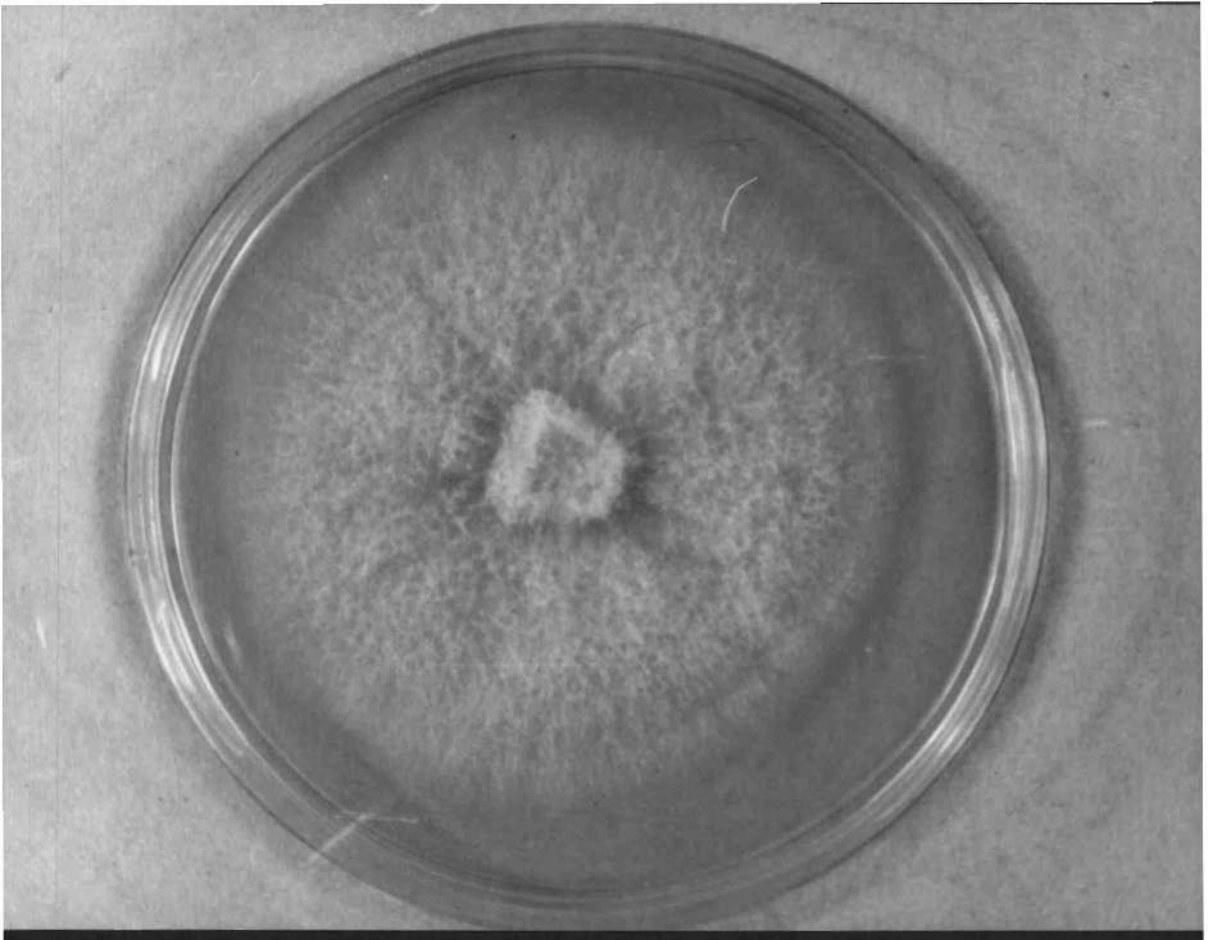


PLATE 8

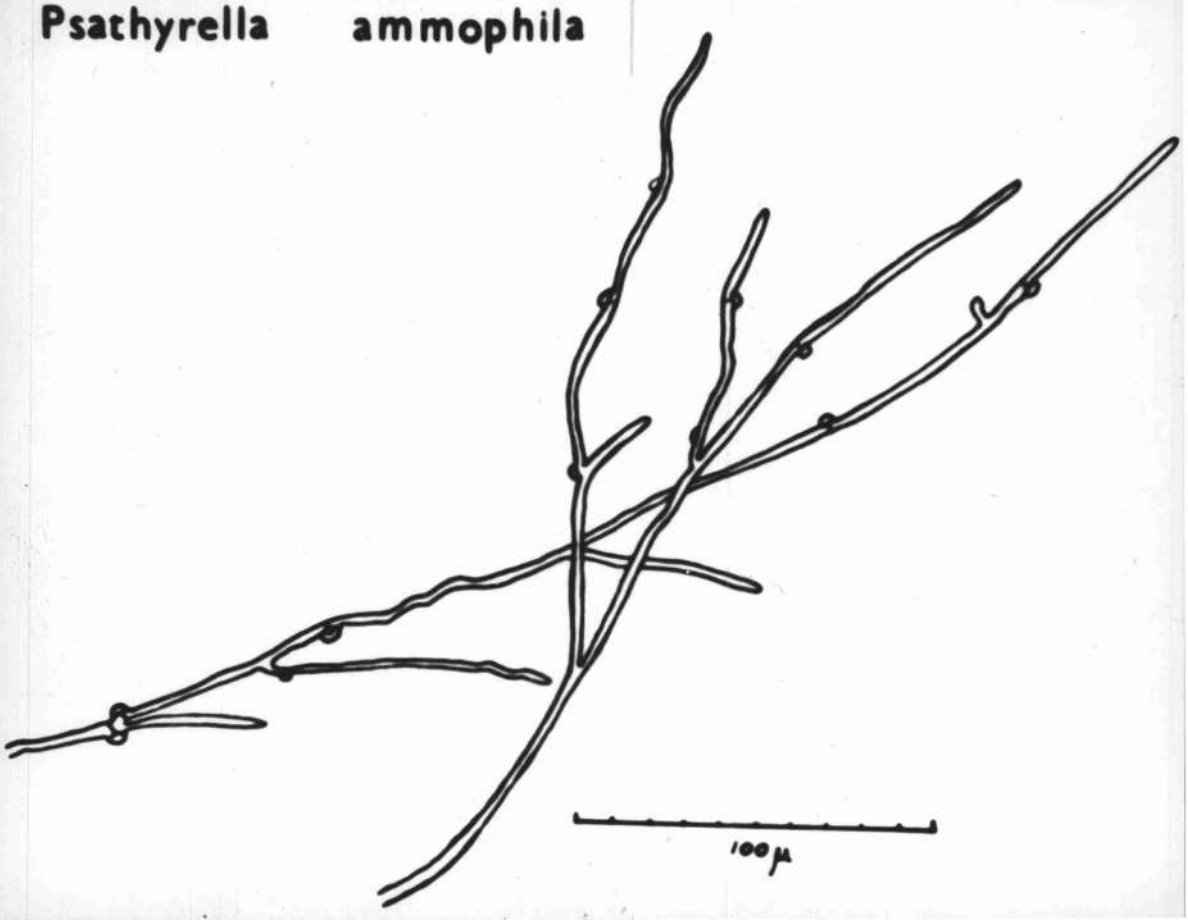
Psathyrella ammophila growing on malt extract agar for two weeks.

PLATE 8a

Camera lucida drawing of Psathyrella ammophila.



Psathyrella ammophila



D. Peziza ammophila

This is a fast growing Discomycete and increases its radius at an average rate of 20mm per week under the conditions described for the Basidiomycetes. Aerial hyphae are short, whitish and thin. Fungal colonies are always zoned on agar plates.

Subcultures had to be made every two weeks: otherwise the fungus failed to grow. Sometimes up to 20 subcultures were made from the same colony and only one or two grew. Sometimes all failed to grow and the isolation was readily lost. This was the case when subcultures were made from colonies two or more weeks old. Either the fungus produces staling products which prevent the hyphae from growing on fresh agar, or the medium is quickly depleted of some essential growth substance which it cannot synthesize for itself.

E. E 1

This is a Basidiomycete which was isolated from roots of Elymus arenarius in September, 1966 using the technique described in Section 2b(iii).

The fungus was confined to the cortical cells of the older parts of the roots both inter- and intra- cellularly. It was never located in root hairs nor on the outside of the roots. In the cortex the hyphae were straight and branched and almost all septa had visible clamp connections.

When this fungus was grown on 2% malt extract agar at 23°C, its rate of growth was very slow reaching an average of 2.0mm per week. At first the hyphae were brownish with very little aerial hyphae. After about three weeks hyphae started growing from the inoculant on top of the previous

growth. This new development was white and compact.

On examining the colony microscopically, it was found that the hyphae in the primary growth were chiefly narrow and straight while those in the secondary growth were bulbous and much broader (Plates 9 and 10).

When roots of E. arenarius, A. arenaria and A. junceiforme were inoculated with this fungus, its morphology was again different (Plate 11). It was much more bulbous than when it was grown in culture.

F. A 1 and A 2

These are two Basidiomycetes which were isolated from Ammophila roots in May, 1967, using the technique described in Section 2b(iii).

As in the case of E 1 the hyphae were confined to the cortical cells both inter- and intra- cellularly and were never found in young roots, in young parts of older roots nor in the small lateral roots. A 1 and A 2 appeared to grow together but A 2 extended further down the length of the root than A 1 which was confined to the upper third of the roots. In Plate 12, A 1 is seen as bulbous hyphae in the cells of the cortex while A 2 is just visible as thin lines. Plates 13 and 14 show more details of their structure.

On malt extract agar at 23°C, A 1 was a relatively slow grower - average increase of 2.6mm per week while A 2 grew at 4.6mm in the same time.

In A 1 the hyphae appeared brownish, no aerial hyphae were visible and microscopically the hyphae were bulbous. Septa and clamp connections were readily seen.

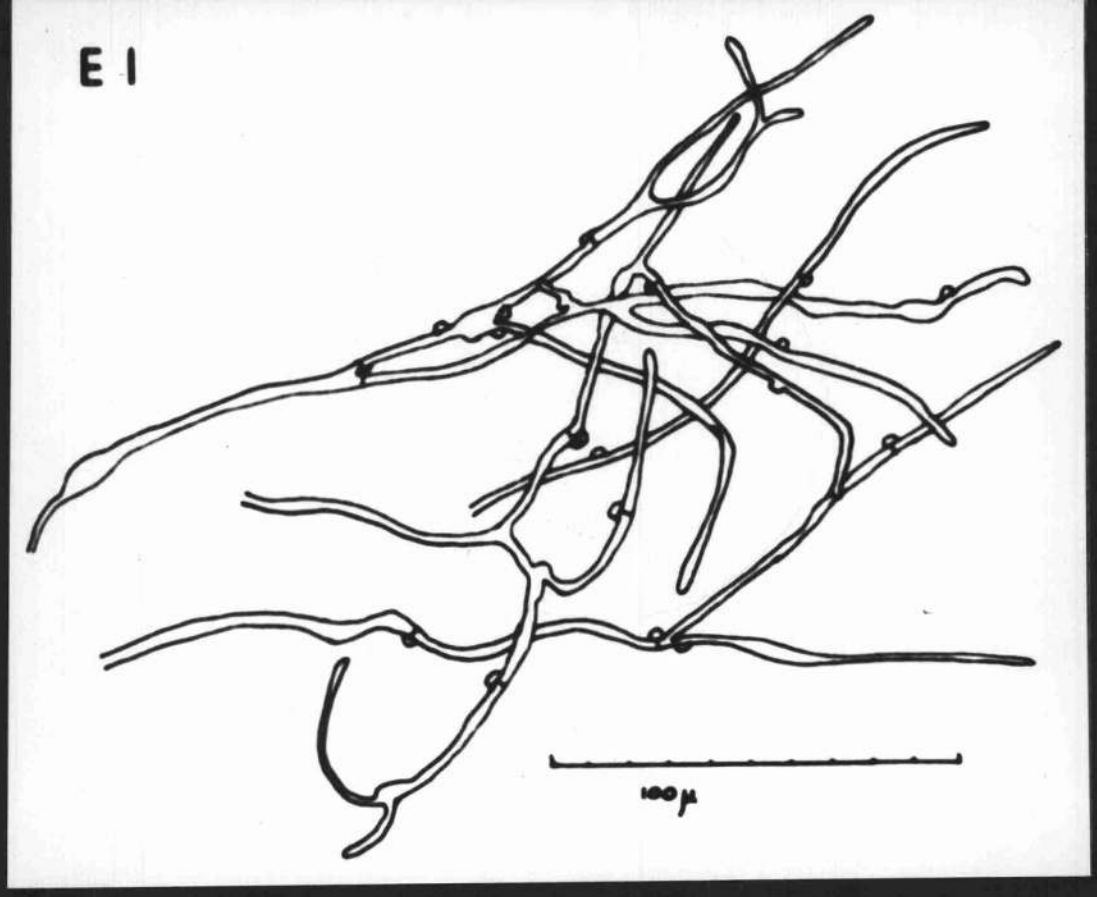
A 1 morphologically resembles E 1. The hyphae of both of them appear brownish, aerial hyphae negligible, on malt extract agar the growth is slow and hyphae are bulbous.

PLATE 9

Camera lucida drawing of hyphal tips of E 1 taken from the growing edge of a three week old colony.

PLATE 10

Camera lucida drawing of hyphal tips of E 1 taken from the older region of the colony (secondary growth).



E 1

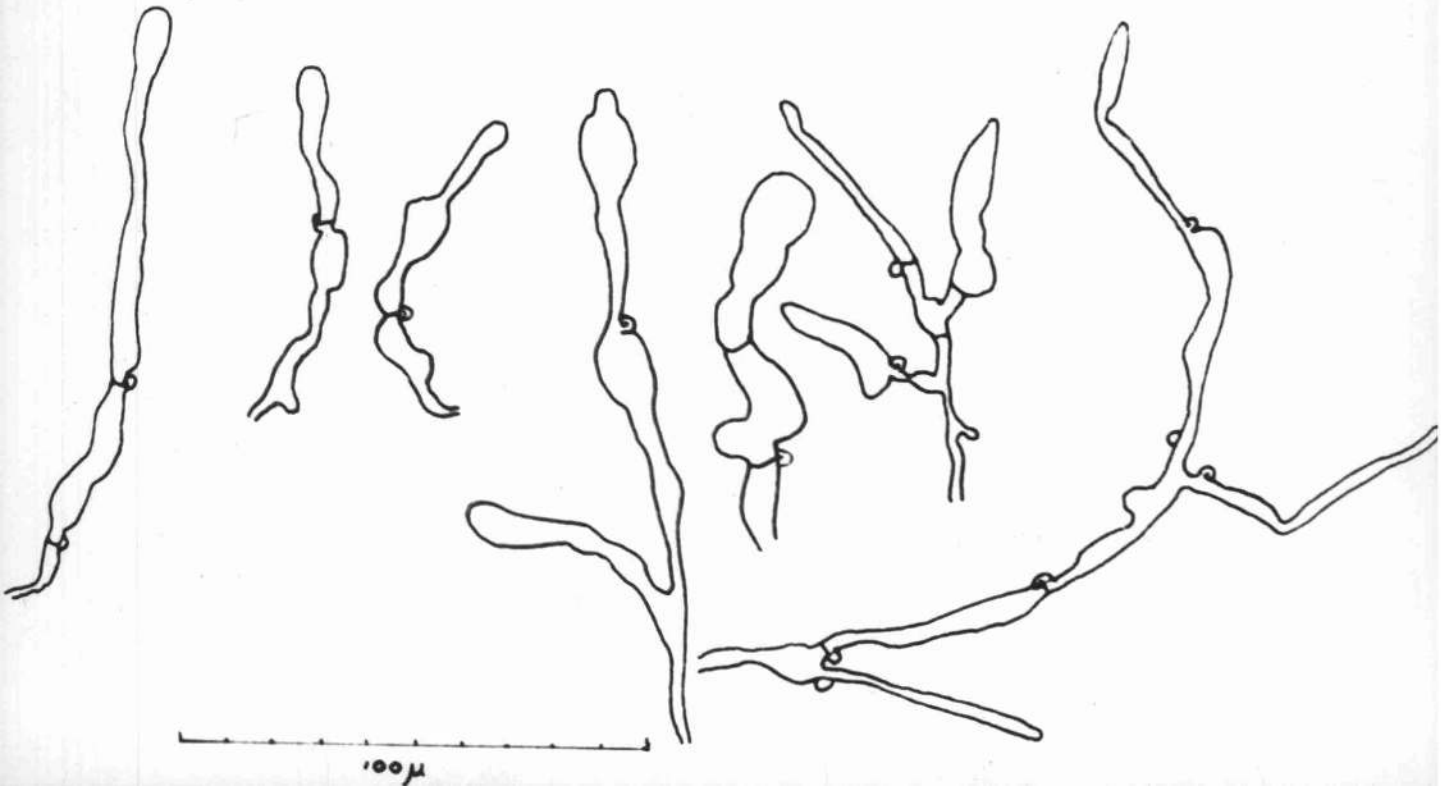
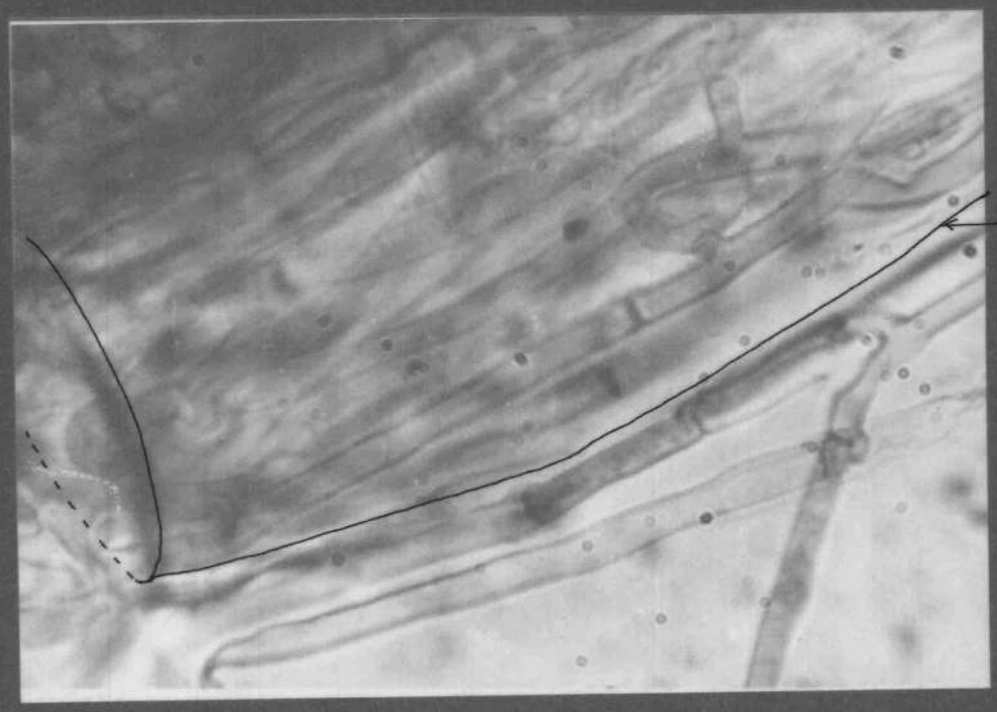


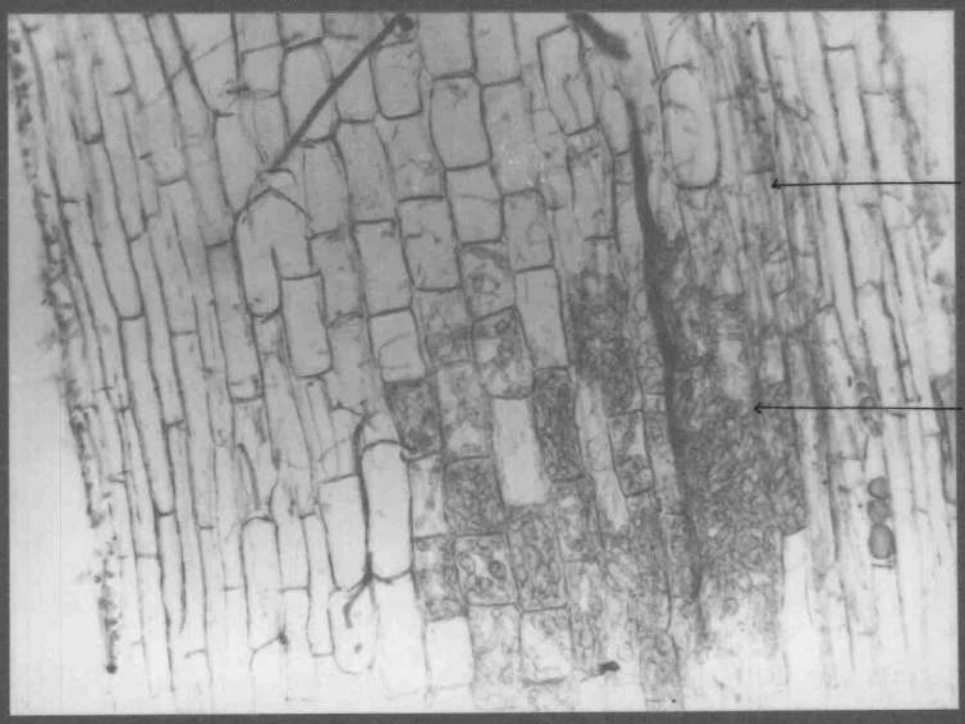
PLATE 11

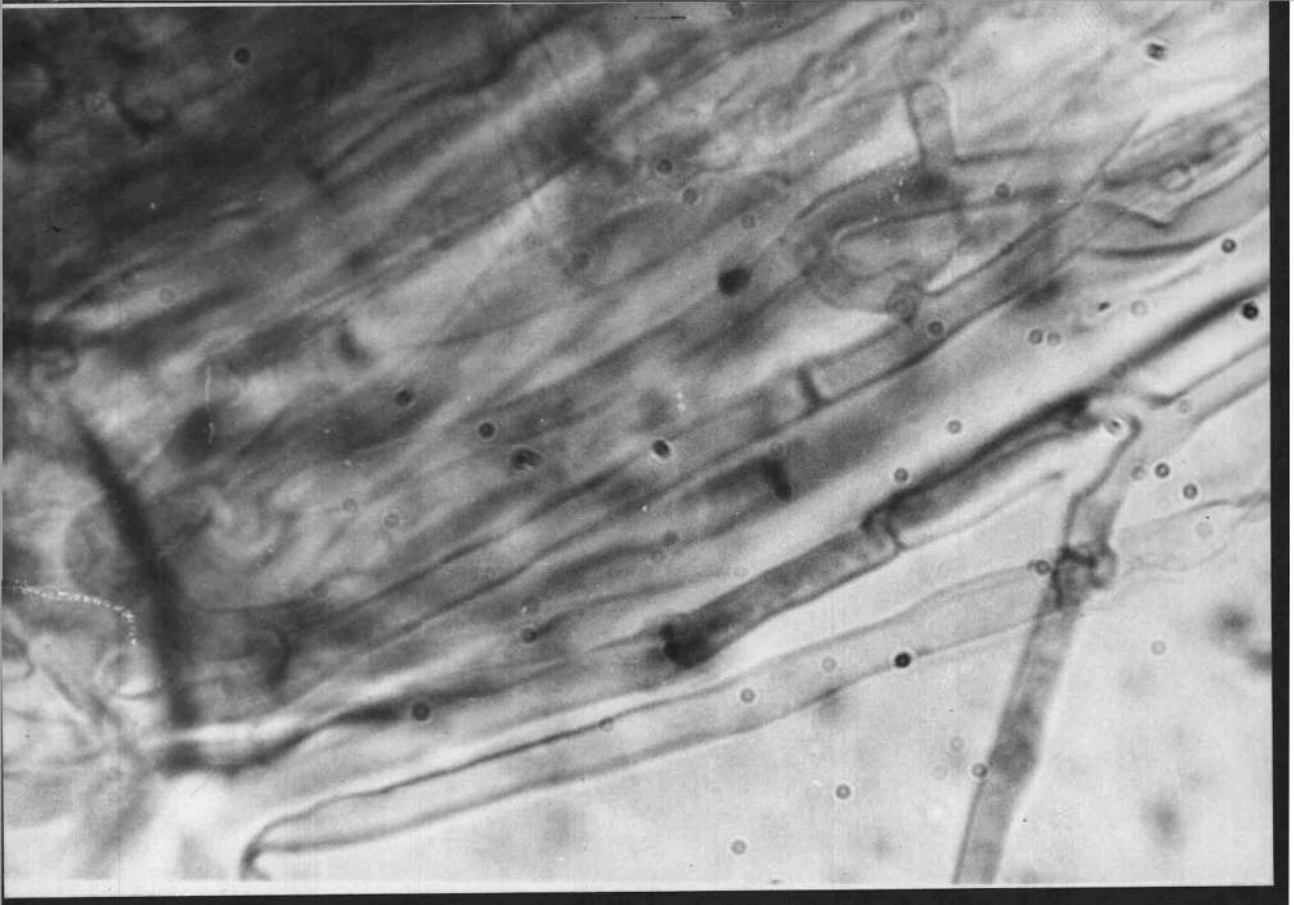
Non - bulbous hyphae of E 1 in the cortical cells of E. arenarius which was collected from Tentsmuir. Compare this with Plate 59 where the hyphae are bulbous. (x 1000).

PLATE 12

A 1 (bulbous) and A 2 (thin and narrow) in cortical cells of A. arenaria taken from Tentsmuir (x100).


OUTLINE OF
THE CELL-WALL.


A 2.
A 1.



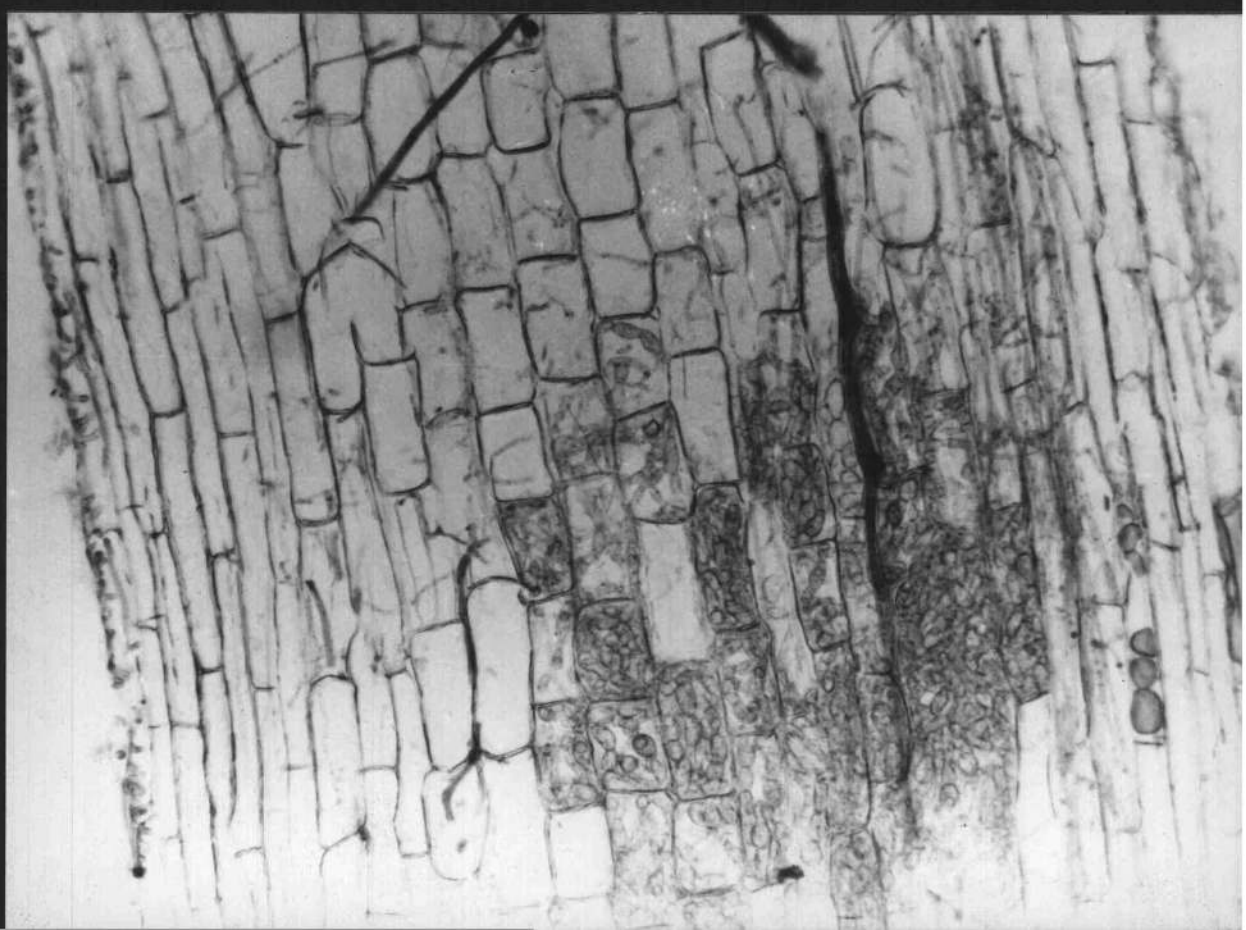
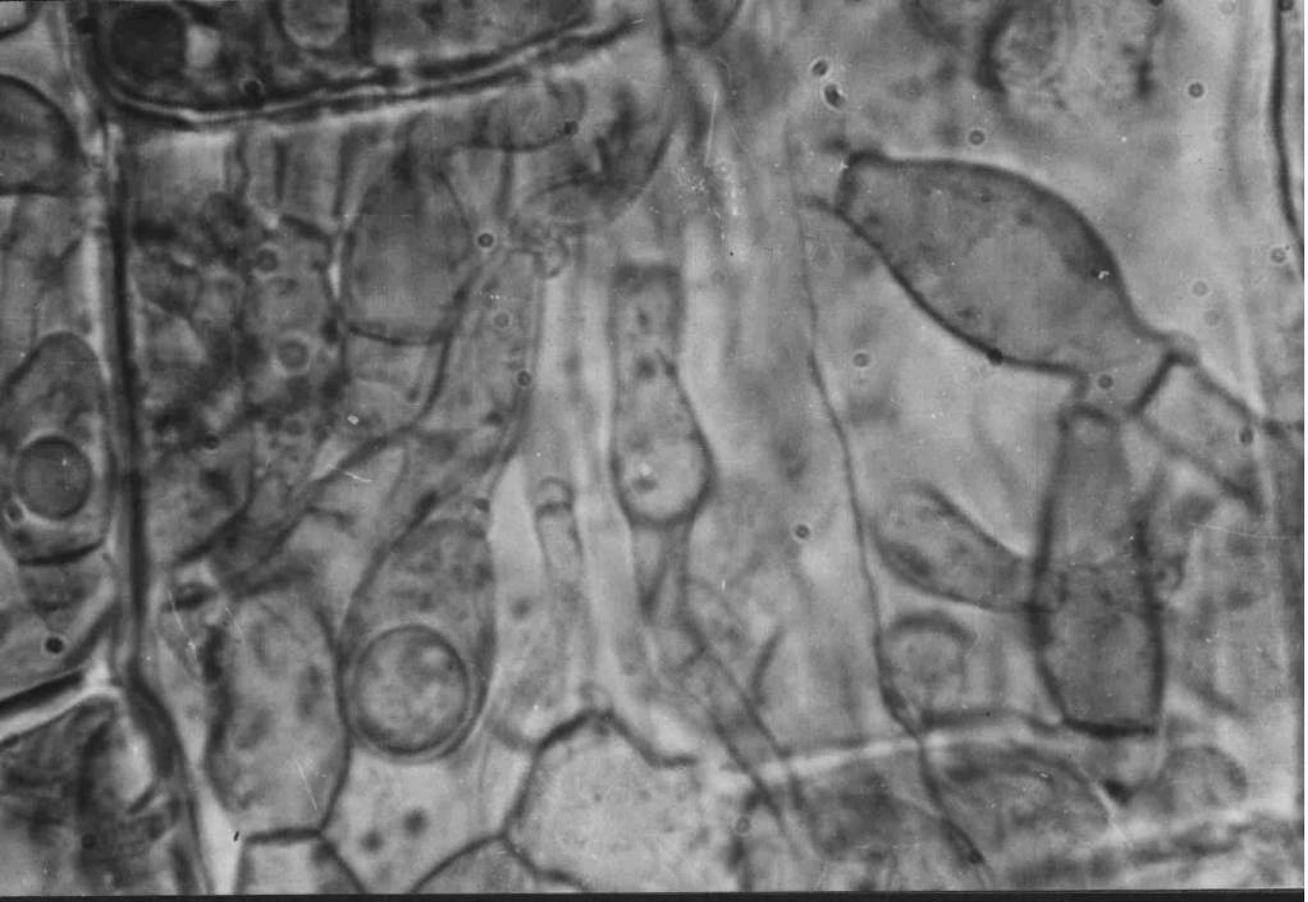


PLATE 13

A 1 in cortical cells of A. arenaria (x1000).

PLATE 13a

Camera lucida drawing of A 1 grown on malt extract agar.



A 1

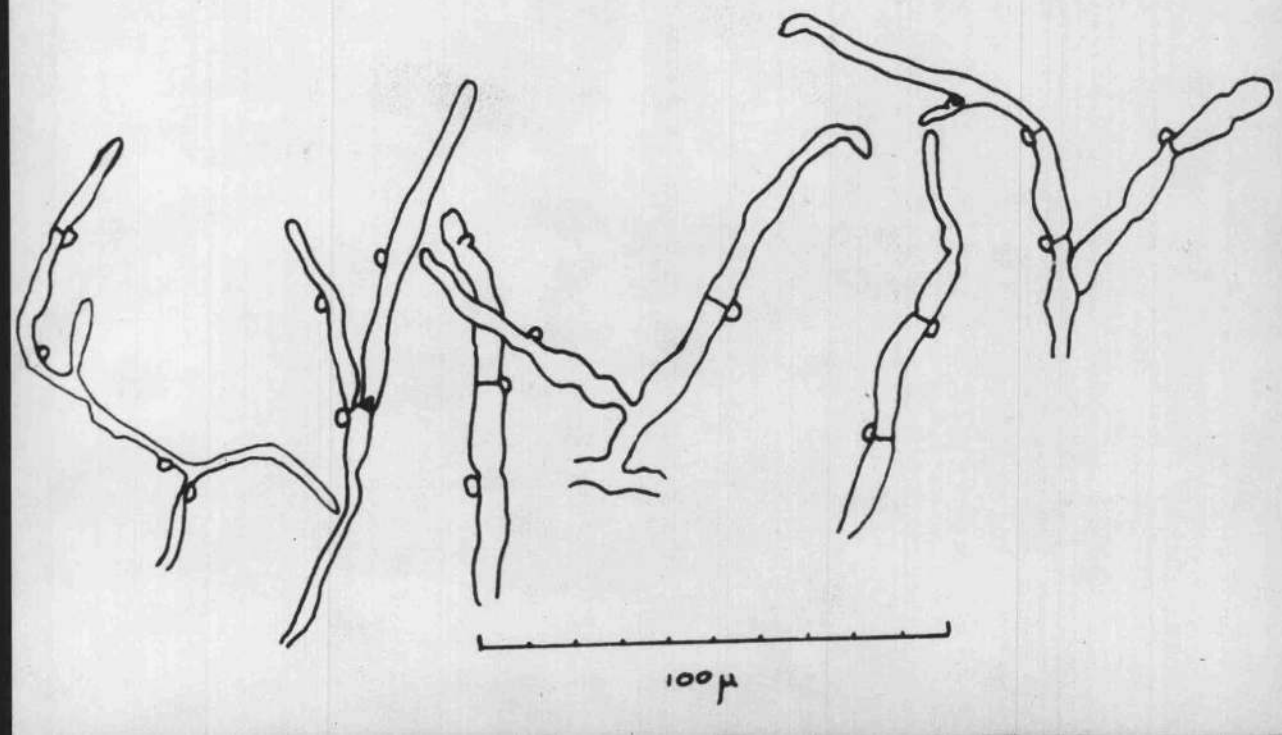
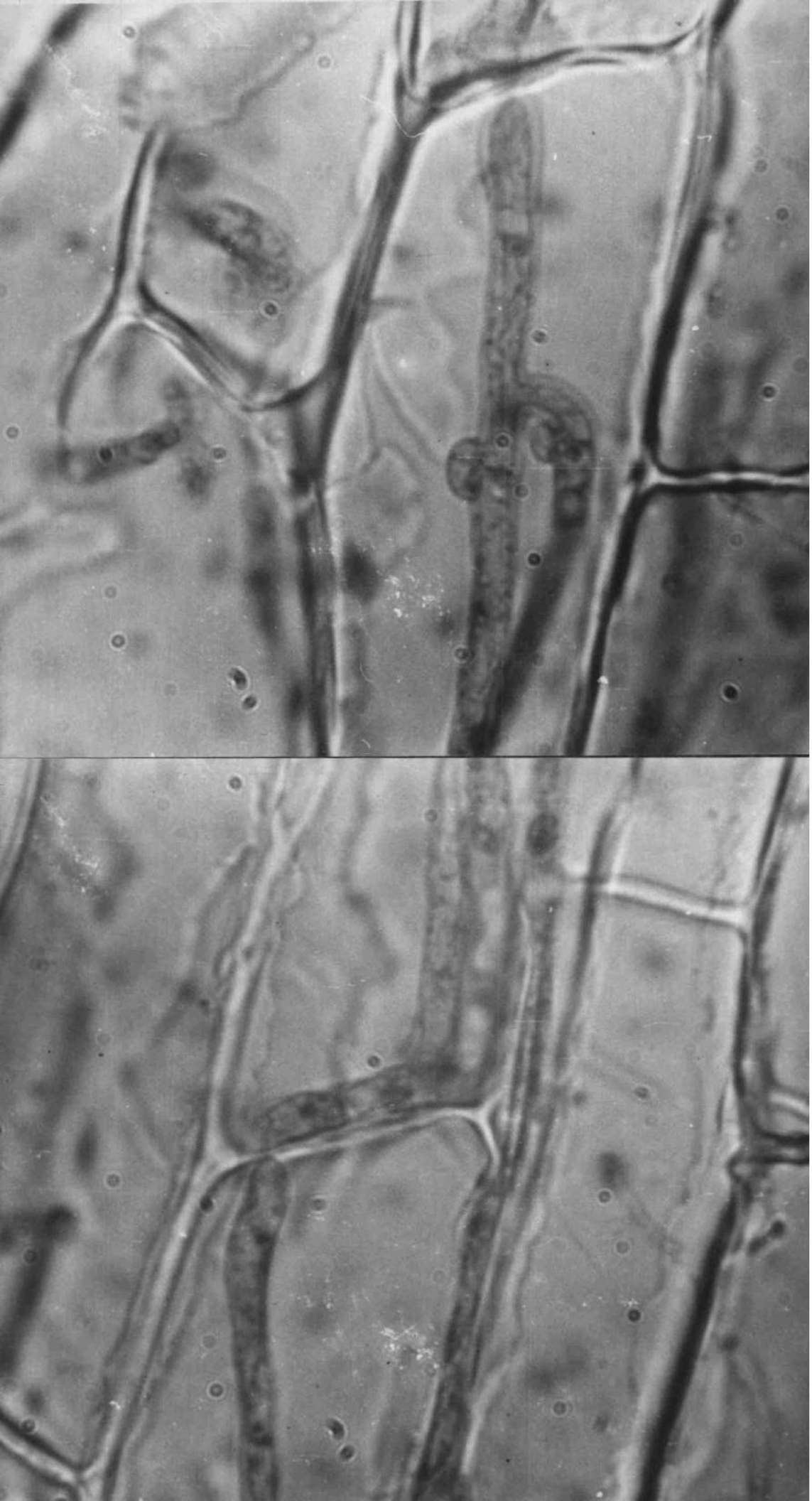
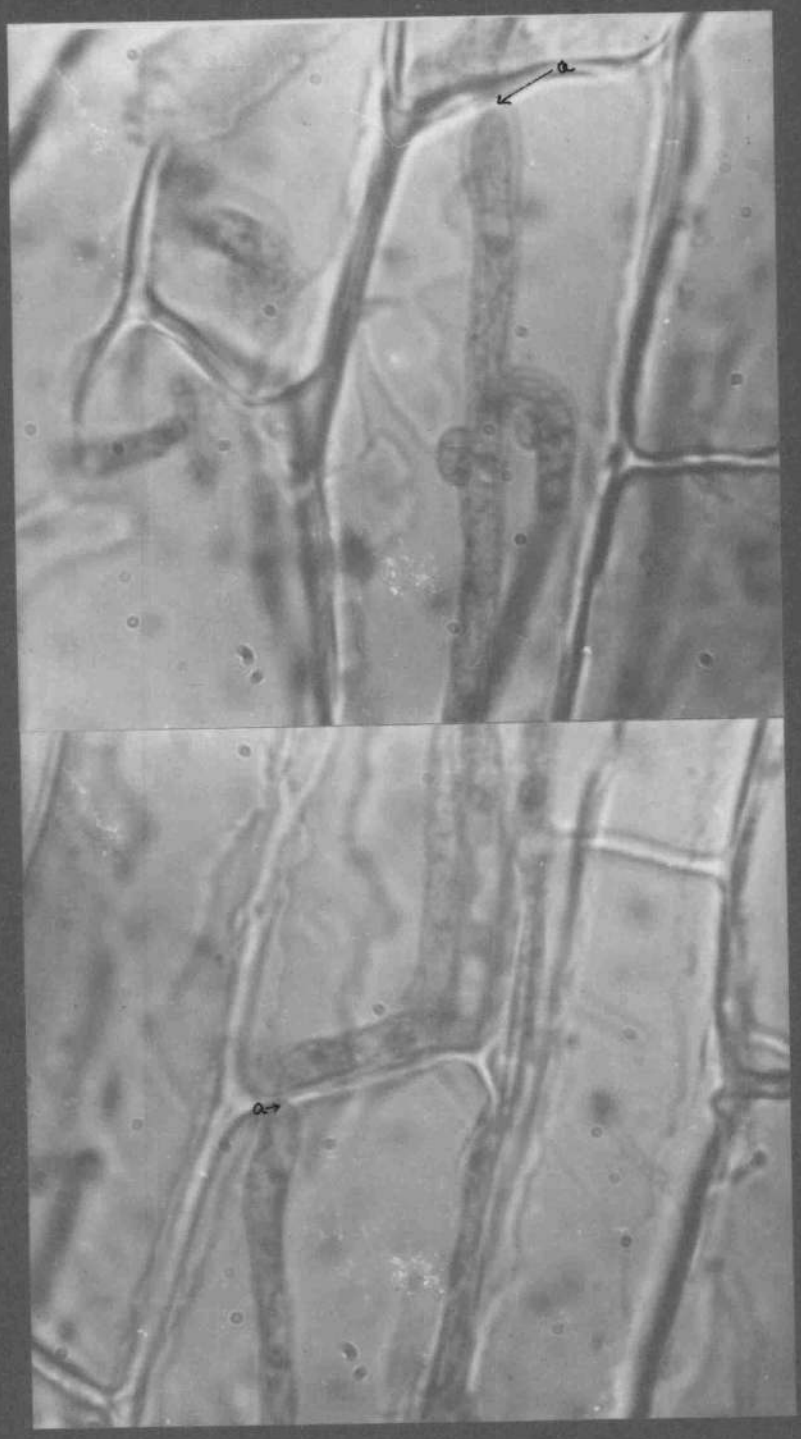


PLATE 14

A 2 in cortical cells of A. arenaria. Hyphae pass from cell to cell by constriction (a),(x1000).




a
a

Physiologically they were alike in their vitamin and nitrogen requirements, the quantities of cellulolytic enzymes produced in vitro and the amount of polyphenol oxidase which they produced on tannic and gallic acid media. However, they differed in the production of pectinolytic enzymes and in the stimulation of their growth by extracts from the roots of A. arenaria, A. juncciforme and E. arenarius. They are probably two strains of one and the same fungus and their difference could be due to the fact that E 1 had been isolated eight months before A 1 and might have undergone changes in its physiology.

Discussion

On 2% malt extract agar C. dunensis, M. grammopodia, E 1 and A 1 invariably produced bulbous hyphae. A 1 also appeared bulbous in situ in the roots of A. arenaria. The hyphae of E 1 were not originally bulbous in the roots of E. arenarius at the time of isolation but when on 2% malt extract agar and when the isolate reinfected the roots of the three grasses, they were bulbous. This is perhaps a phenomenon which requires further mention.

Watling (personal communication, 1967) has found that the bulbousness of bolbitiaceous species increased with the amount of sucrose incorporated in the medium. He suggests that it is related to osmotic pressure. As no experimental data is available it cannot be stated that his explanation applies to these fungi under investigation.

Swollen or bulbous hyphae have been reported at least in two other groups of fungi i.e. Coprinus and Penicillium. Madelin (1960) offered an explanation for the occurrence of swollen cells in the vegetative mycelium of Coprinus lagopus. When this fungus had been growing for eight days,

a very large proportion of the cells within the agar at distances greater than 5mm from the margin were variously inflated and grossly distorted and were completely filled with dense and highly refractive contents. Despite the swelling, clamp connections were generally clearly visible. (This also holds true for Conocybe dunensis, for A 1 and for E 1). These inflated cells were absent in aerial hyphae.

Between the 11th and the 16th days, when sporophores were growing and maturing, Madelin observed visible changes within the swollen cells - vacuoles appeared and grew progressively larger while the amount of deeply staining cytoplasm proportionally diminished. The spent cells retained their shapes but lost their staining property. These changes were also observed on haploid mycelium which usually fruited tardily and abnormally, if at all. As a result of these observations, Madelin suggested that the swollen cells played an important part in reserving food for fruiting.

Conocybe dunensis produced fruit body primordia on malt extract agar after about three weeks but only small abnormal fruit bodies have been produced on this medium. Swollen hyphae were observed almost as soon as they started growing. So far, the other fungi with bulbous hyphae have not been observed to fruit in culture. Perhaps they can go as far as storing up food reserves but the medium lacks some factor which they need for fruiting.

Bent and Morton (1963) observed swollen hyphae in submerged culture of Penicillium griseofulvum. Their formation was regarded by Lilly and Barnett (1951) as response to one of several conditions unfavourable to growth e.g. low pH, potassium and oxygen deficiency, high osmotic pressure or the

presence of inhibitors. Bent and Morton (1963) found that the "large cells were morphologically and developmentally equivalent to sporophores in which the normal development leading to conidial formation is not completed owing to some metabolic derangement caused by an abnormally low pH of the medium".

Although the relationship between large cells and sporophore production appears to provide satisfactory explanation in those species studied, it cannot be assumed that this is true in every case or that the large cells are morphologically equivalent in all fungi.

SECTION 4

PHYSIOLOGICAL CHARACTERISTICS OF THE ISOLATES

4a Vitamin requirement

This experiment was conducted with a view to ascertaining whether the fungi were able to synthesize their own vitamins or whether these had to be supplied from an external source. The following fungi were subjected to the test:- C. dunensis, M. grammopodia, Psathyrella ammophila, Peziza ammophila, E 1, A 1 and A 2. They were grown for 2-3 weeks on a medium containing 2% difco agar and 1% glucose to eliminate the possibility of them carrying over traces of vitamins with them.

Method

The vitamins thiamine (100 µg/litre), biotin (5 µg/litre) and pyridoxine (100 µg/litre) for which filamentous fungi are most frequently deficient, were supplied to seven series of culture media in the following manner:-

Solution 1 = basal medium acting as a control
" 2 = " " + thiamine (B.D.H:LGR)
" 3 = " " + biotin (B.D.H:LGR)
" 4 = " " + pyridoxine (B.D.H:LGR)
" 5 = " " + thiamine and biotin
" 6 = " " + thiamine and pyridoxine
" 7 = " " + biotin and pyridoxine
" 8 = " " + thiamine, biotin, pyridoxine

The basal synthetic culture medium was the glucose - asparagine medium of Lilly and Barnett (1951) and had the following constituents:-

Glucose	20.0 grams	
Asparagine	2.0 "	(B.D.H:LGR)
KH_2PO_4	1.0 "	(B.D.H:LGR)
$MgSO_4.7H_2O$	0.5 "	
Fe^{+++}	0.2 mg	
Zn^{++}	0.2 mg	
Mn^{++}	0.1 mg	
Distilled water	1000 ml	

pH adjusted to 6 before sterilization

The basal medium was sterilized and when cooled the vitamins were added at the rate indicated above. The thiamine and pyridoxine were made up in 70% alcohol (stock solutions contained 10mg/100ml) and stored in a refrigerator until needed. When 20% alcohol (Lilly and Barnett, 1951) was used, contamination was observed after the flasks with the inoculants had been incubated for eight days. There was no such problem when 70% alcohol was used instead of 20%. The biotin was bought in 1ml ampoules containing 1 µg. The media were dispensed in 25ml quantities in the previously sterilized 100ml Erlenmeyer flasks and were inoculated and incubated at 23°C. The experiment was set up in duplicate.

In this, as in all quantitative experiments in this investigation, equal size inoculants were used. A 5mm square was outlined on a sheet of graph paper. Each petri plate from which the inoculants were taken, was superimposed on the graph paper so that the growing edge of the colony coincided with one side of the square. The inoculants were then cut out 5mm square and approximately 2mm deep.

After ten weeks the mycelia were rinsed in distilled water, transferred to previously weighed aluminium foil boats and dried at 50°C to constant weight. Their dry weights were then determined.

Results

The results shown in Table 2 have been calculated as percentages of the control and are expressed in Plates 15–21. From Table 2 and Plate 15, it can be seen that C. dunensis is able to grow on the basal medium but the addition of thiamine and pyridoxine, singly or together, caused a marked increase in growth. Biotin alone caused a decrease in growth, but the addition of thiamine was effective and doubled the growth. When biotin and pyridoxine were in the same medium, growth was equivalent to that on the basal medium. When all three vitamins were together, growth was as good as with thiamine on its own but slightly less than with pyridoxine alone, thiamine + pyridoxine or with thiamine and biotin together.

It, therefore, appears that thiamine and pyridoxine must be added to the substrate for the healthy growth of C. dunensis but that biotin on its own or with pyridoxine has an inhibitory effect.

In M. grammopodia, the basal medium permitted some growth but the addition of thiamine caused almost three-fold increase. When biotin and thiamine were incorporated, growth was less than that on the basal medium but when pyridoxine was added to these two there was slight increase in growth over that on the basal medium.

TABLE 2

Vitamin deficiency - done in duplicate

Mean dry weight in mg. Grown for 10 weeks. Dried at 50°C.

Fungi / Solution	Conocybe	Peziza	Melanoleuca	Psathyrella	A 1	A 2	E 1
Minimal medium (mm)	321.1	17.0	38.4	13.9	87.1	58.0	59.5
mm + thiamine (t)	506.0	50.8	87.0	100.5	112.0	166.4	136.5
mm + biotin (b)	201.3	60.8	75.5	37.5	77.9	54.4	46.7
mm + pyridoxine (p)	513.8	16.8	20.3	24.9	117.8	59.0	80.3
mm + t + b	534.7	27.9	36.0	24.5	122.3	169.0	143.0
mm + t + p	534.8	25.8	47.7	15.1	181.9	159.9	126.1
mm + b + p	328.2	20.5	53.4	15.8	81.4	89.8	61.5
mm + t + b + p	496.7	62.3	40.0	42.6	95.8	173.0	84.9

Biotin, by itself, permitted growth which was nearly twice that on the basal medium. Growth was reduced to nearly half that on the basal medium when pyridoxine alone was added.

Therefore, M. grammopodia grows better with an external supply of thiamine or biotin but it would appear that pyridoxine interfered with the stimulatory effect of biotin when they were both in the same medium.

Psathyrella ammophila grew very poorly on the basal medium but the addition of thiamine greatly increased the growth to more than sevenfold. Biotin by itself was effective in causing nearly threefold increase whereas pyridoxine permitted only twofold increase. Thiamine and biotin together were as effective as pyridoxine alone but when all three were together growth was stimulated to three times that on the basal medium. Thiamine + pyridoxine permitted growth which was just better than that on the basal medium but slightly poorer than pyridoxine + biotin.

Growth of Psathyrella ammophila is, therefore, stimulated by an external supply of each of the three vitamins particularly by thiamine.

Peziza ammophila

The results also show that the basal medium supported some growth. Both thiamine and biotin added singly, markedly increased the growth, biotin more so than thiamine; but pyridoxine had no effect. When any two of the vitamins were added to the medium, the amount of growth was slightly increased over that of the basal medium but all three together permitted the best growth of all.

This fungus also requires an external supply of thiamine and biotin in order for it to produce good growth. It is probably able to synthesize its own pyridoxine.

A 1

The basal medium supported very good growth with this fungus although the addition of thiamine or pyridoxine produced an increase. When biotin was the only vitamin present growth was depressed. The addition of thiamine plus biotin gave better growth than that obtained when they were added singly. Thiamine plus pyridoxine had a combined effect of increasing the growth to more than twice that obtained on the basal medium. The combination of biotin with pyridoxine had a reducing effect but when thiamine also was added growth increased.

Biotin, therefore, seems to have an inhibitory effect while thiamine and pyridoxine stimulated growth.

A 2

In A 2, thiamine, whether singly or combined, markedly increased the growth. Biotin caused a decrease while pyridoxine had no effect. Growth increased when pyridoxine and biotin were added together.

The growth of this fungus is, therefore, stimulated by an external supply of thiamine.

E 1

E 1 grew on the basal medium but the addition of thiamine permitted much better growth. Biotin was inhibitory but the addition of thiamine to biotin gave better growth than was obtained with thiamine alone. Pyridoxine when added singly caused an increase in growth but with thiamine the growth was increased to more than

PLATE 15

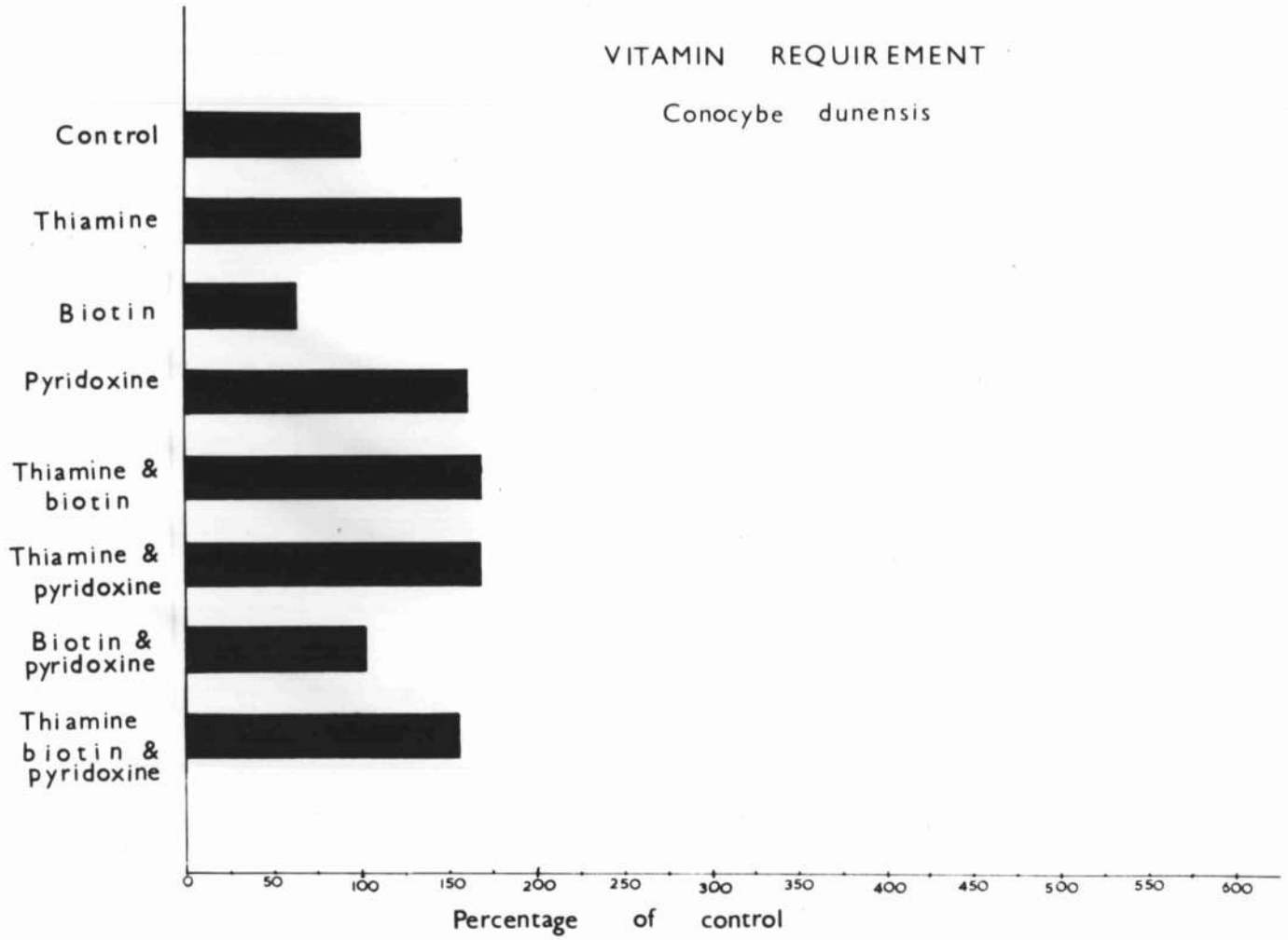

VITAMIN REQUIREMENT
Conocybe dunensis
Control
Thiamine
Biotin
Pyridoxine
Thiamine & biotin
Thiamine & pyridoxine
Biotin & pyridoxine
Thiamine biotin & pyridoxine
0
50
100
150
200
250
300
350
400
450
500
550
600
Percentage of control

PLATE 16

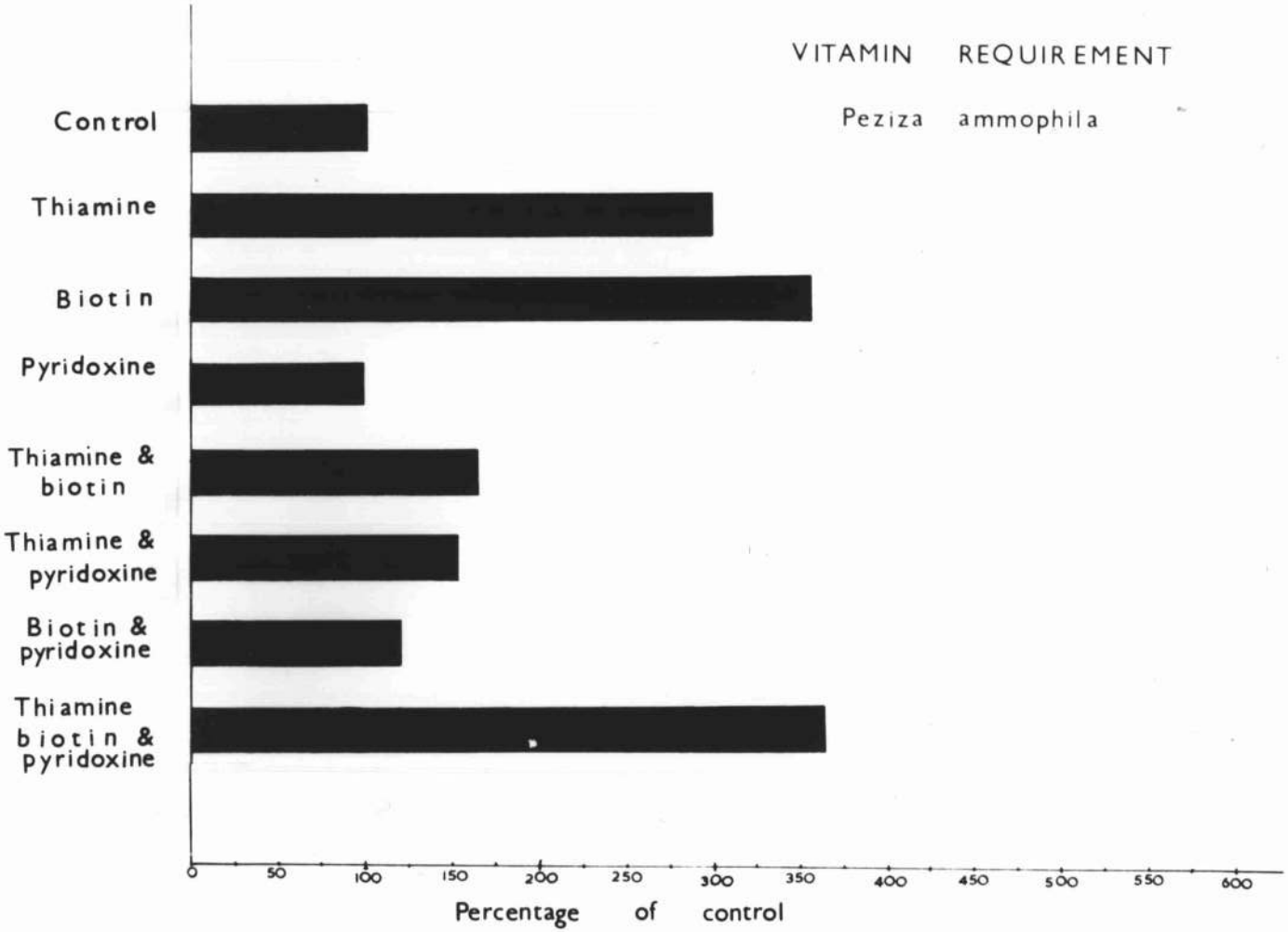

VITAMIN REQUIREMENT
Peziza ammophila
Control
Thiamine
Biotin
Pyridoxine
Thiamine & biotin
Thiamine & pyridoxine
Biotin & pyridoxine
Thiamine biotin & pyridoxine
0
50
100
150
200
250
300
350
400
450
500
550
600
Percentage of control

PLATE 17

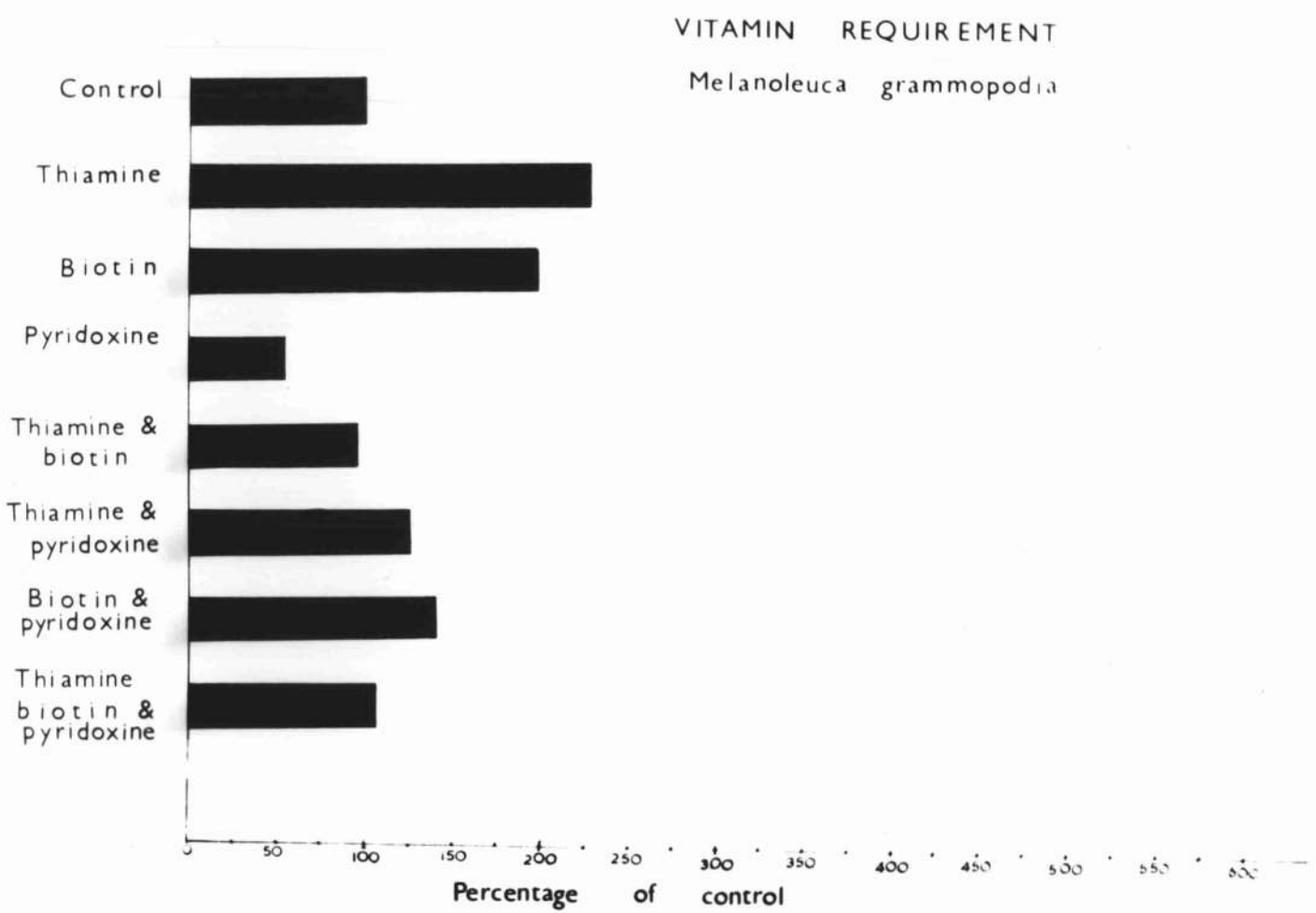

VITAMIN REQUIREMENT
Melanoleuca grammopodia
Control
Thiamine
Biotin
Pyridoxine
Thiamine & biotin
Thiamine & pyridoxine
Biotin & pyridoxine
Thiamine biotin & pyridoxine
0
50
100
150
200
250
300
350
400
450
500
550
600
Percentage of control

PLATE 18

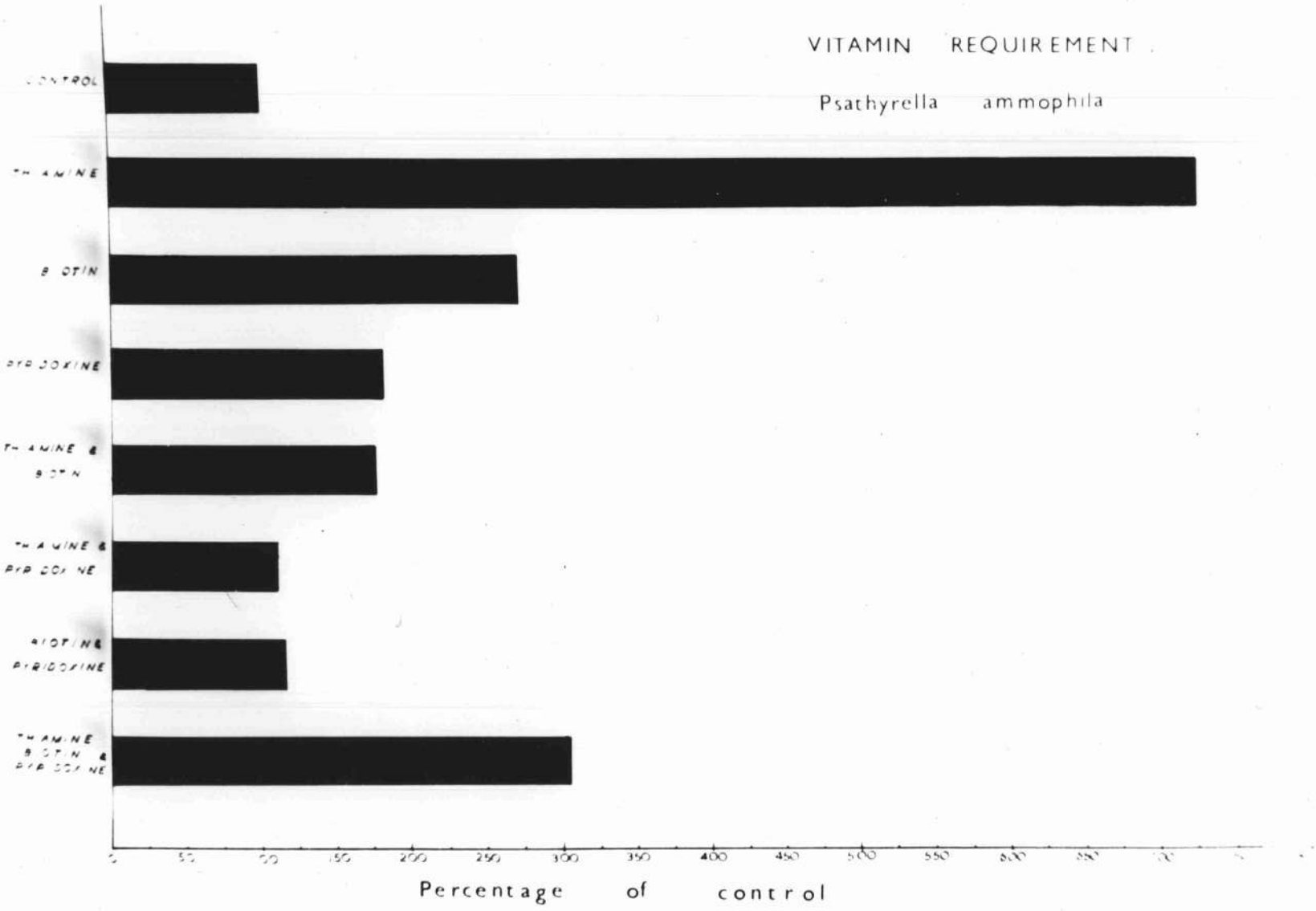
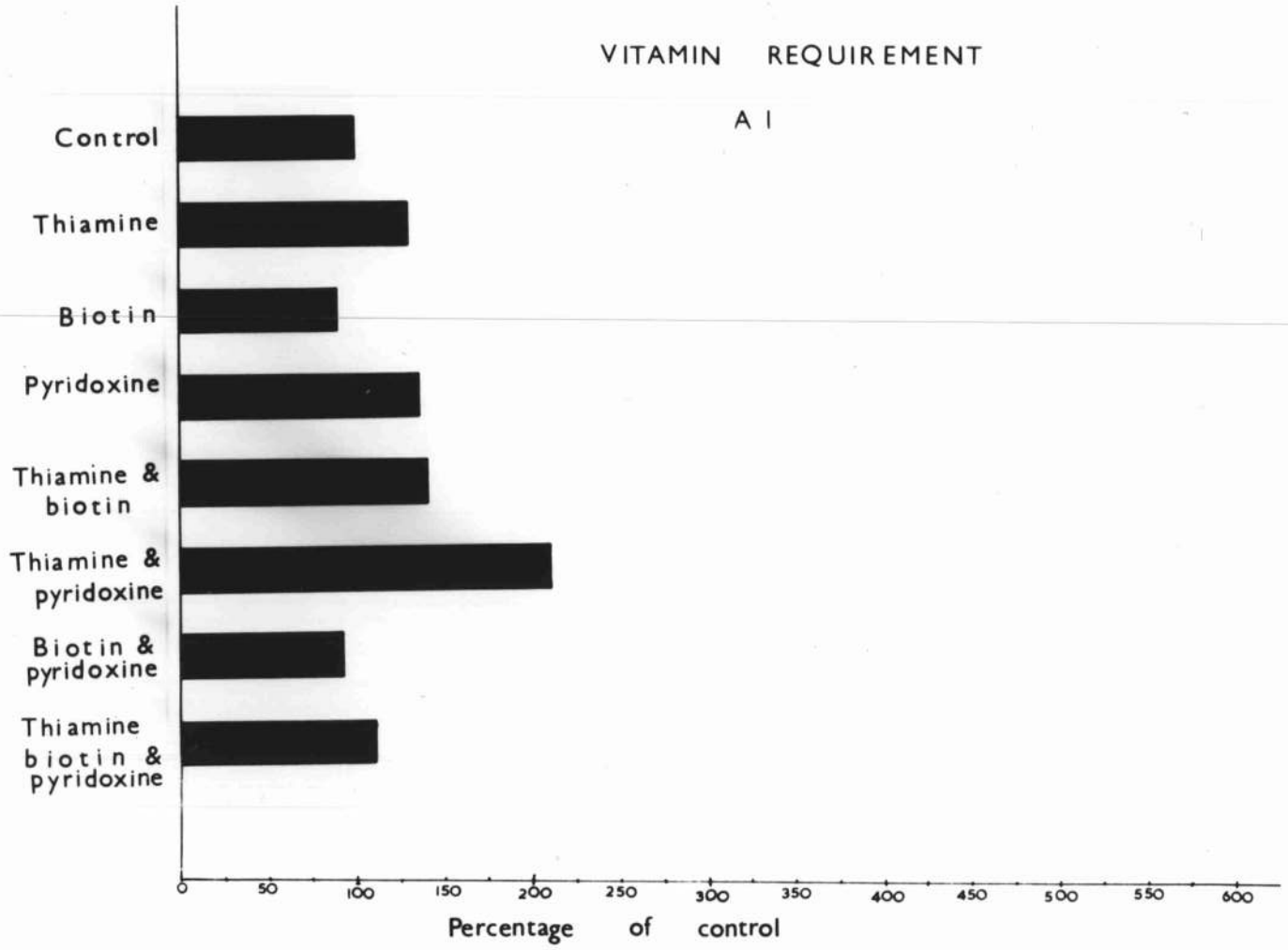

VITAMIN REQUIREMENT
Psathyrella ammophila
CONTROL
THIAMINE
BIOTIN
PYRIDOXINE
THIAMINE & BIOTIN
THIAMINE & PYRIDOXINE
BIOTIN & PYRIDOXINE
THIAMINE BIOTIN & PYRIDOXINE
Percentage of control

PLATE 19


VITAMIN REQUIREMENT
A I
Control
Thiamine
Biotin
Pyridoxine
Thiamine & biotin
Thiamine & pyridoxine
Biotin & pyridoxine
Thiamine biotin & pyridoxine
0
50
100
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200
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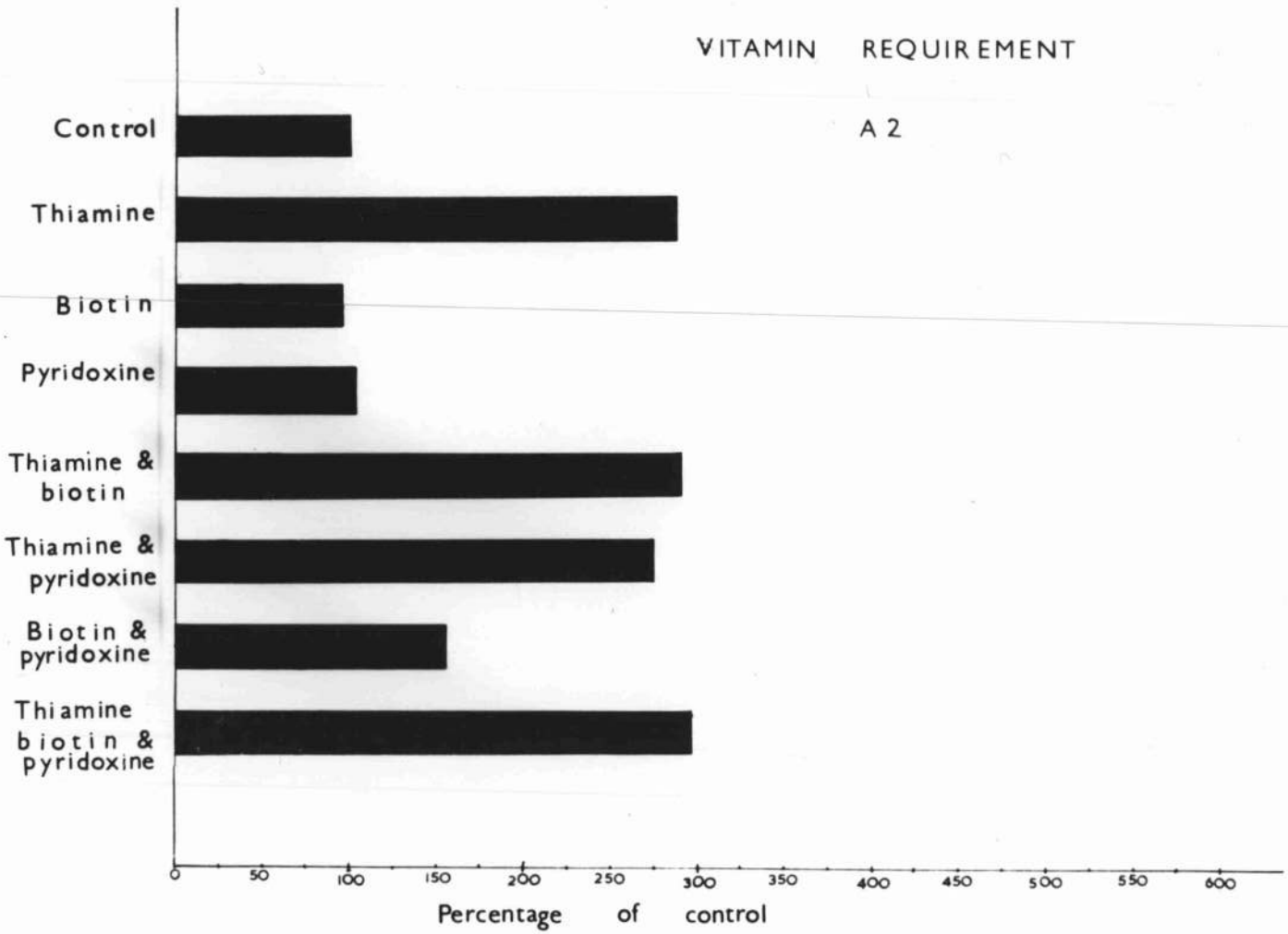
400
450
500
550
600
Percentage of control

PLATE 20


VITAMIN REQUIREMENT
A 2
Control
Thiamine
Biotin
Pyridoxine
Thiamine & biotin
Thiamine & pyridoxine
Biotin & pyridoxine
Thiamine biotin & pyridoxine
0
50
100
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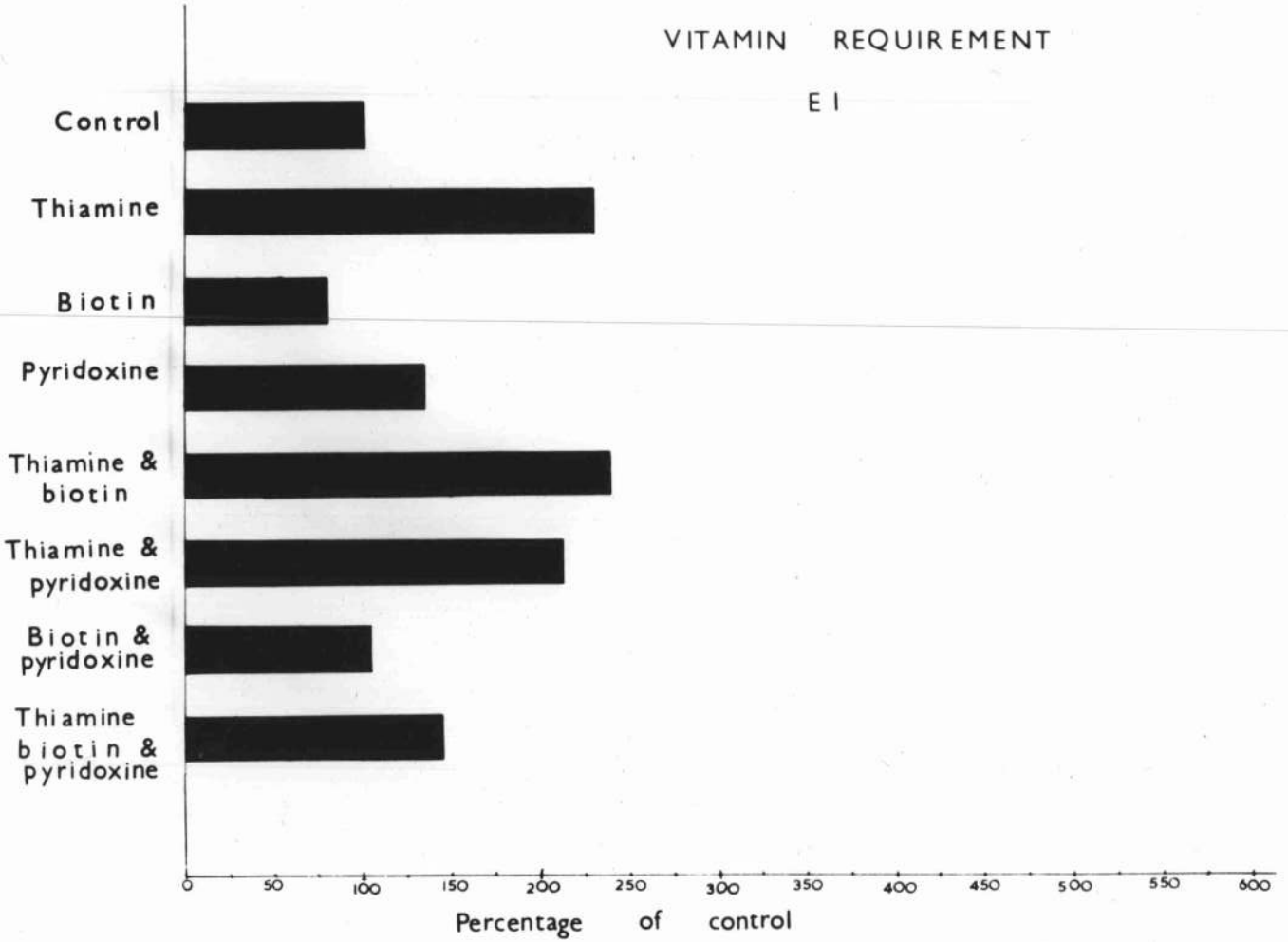
200
250
300
350
400
450
500
550
600
Percentage of control

PLATE 21


VITAMIN REQUIREMENT
E I
Control
Thiamine
Biotin
Pyridoxine
Thiamine & biotin
Thiamine & pyridoxine
Biotin & pyridoxine
Thiamine biotin & pyridoxine
0
50
100
150
200
250
300
350
400
450
500
550
600
Percentage of control

twice that on the basal medium. Again pyridoxine had little effect when combined with biotin but all three together permitted some growth.

This fungus, therefore, grows much better when there is an addition of thiamine.

Discussion

Fungi require vitamins for growth, reproduction and other vital functions. Certain ones must obtain one or more of the vitamins from the substrate, e.g. Phycomyces blakesleeanus requires thiamine; others are able to synthesize from the compounds of the medium all the vitamins in sufficient quantities to meet their needs e.g. Aspergillus niger. From the experiment described above, it can be seen that all seven fungi responded by increased growth to addition of thiamine, four of them to addition of biotin and only two to pyridoxine.

This experiment, however, was only a "screening test" and the results need to be verified under more controlled conditions. For example, the chemicals should be purified prior to use to avoid the risk of contamination by significant amounts of the vitamins. The experiment should also run over a considerable period of time because fungi change during development in their synthetic capacity and in their vitamin needs (Cochrane, 1958).

Another problem is that determination should be made over a range of concentration of the vitamins. It has been assumed here that optimum or near optimum amounts of the vitamins were added to the media. The concentrations used were those which have been found to be

near optimum for many filamentous fungi (Lilly and Barnett, 1951). However, the optimum amount of a vitamin may vary with changes in other conditions and may also be different for different fungi.

The experiment also suggests that biotin had a depressing effect on growth of C. dunensis, E 1, A 1 and A 2 and pyridoxine on the growth of M. grammopodia. This may be due to an excessive dosage of the vitamin (Mathur, Barnett and Lilly, 1950; Lilly and Barnett, 1951) or the result may be influenced by some environmental condition. It was found (Barnett and Lilly, 1948) that in Sclerotinia camelliae the same concentration of inositol which stimulated growth at 26°C caused an inhibitory effect at 27°C. Biotin and pyridoxine were also found to reduce the growth of Diplocarpon rosea (Shirakawa, 1955). Shirakawa found that the addition of biotin and pyridoxine, singly or in combination, to a medium containing thiamine depressed growth unless inositol was present. D. rosea is totally deficient for thiamine and partially deficient for inositol. Fries (1943) tested the effect of a very strong pyridoxine concentration on the growth of various Ascomycetes, but no toxic effect could with certainty be proved in any case.

The depressing effect of biotin and pyridoxine in the current investigation might, therefore, be due to an excessive dosage, or to other environmental conditions e.g. temperature. Again, growth inhibitors might have been present in the chemicals used in the basal media and they could have interfered with the effectiveness of the specific vitamins.

For maximum growth to take place, there must be a balance between nutritional and environmental factors and the different vitamins, since growth is a result of interacting factors among which are the vitamins. Vegetative growth measured by dry weight is apparently the most useful criterion of the utilisation of vitamins although reproduction and other processes are also affected and the fungi might exhibit their deficiencies for particular vitamins during these processes rather than in their vegetative stage.

The vitamins required by these fungi as demonstrated by this investigation are probably supplied by the living roots in association with which they grow or from the decomposing plant and animal materials present in the sand. The fungi might also be able to synthesise their own supply of vitamins when in their natural environment although unable to do so under the conditions of the present experiment. It cannot be overemphasized that the pattern of growth exhibited by these fungi might be completely different when the experimental conditions are varied. The seeming vitamin deficiencies must be confirmed using different conditions before they can be regarded as true.

4b Utilization of Nitrogen Sources

This experiment was carried out to determine which compound(s) could be utilized as source(s) of nitrogen by C. dunensis, M. grammopodia, Psathyrella ammophila, Peziza ammophila, E 1, A 1, Polyporus betulinus and Panaeolus semi-ovatus, the last two being used as test fungi. These were chosen because they have quite different nutritional requirements from the other fungi and they were readily available. P. betulinus was kindly supplied by the Botany Department culture store and P. semi-ovatus (wild type) was supplied by Mrs. Krinos in the Honours Class in the same department.

Method

The fast growing members, that is, Peziza ammophila, Psathyrella ammophila, Polyporus betulinus and Panaeolus semi-ovatus were grown for one week on malt extract agar at 23°C while the others were cultured for up to three weeks before they were used as inoculants.

Seven nitrogen sources (Table 3) were separately incorporated in the basal medium suggested by Lilly and Barnett (1951) which contained no chemically combined nitrogen other than any impurities which might be present in the constituents used. The weights of the compounds used were calculated to contain a weight of nitrogen equivalent to that in 2 grams of asparagine (Lilly and Barnett, 1951).

The experiment was set up in duplicate and the media were dispensed in 25 ml quantities in previously sterilized 100 ml Erlenmeyer flasks. The medium was adjusted to pH 6 before sterilization.

The equal sized inoculants were transferred aseptically to the liquid media and allowed to float so that

TABLE 3

Nitrogen sources		Weight in grams/litre
Potassium nitrate	KNO_3	2.69
Ammonium tartrate	$(NH_4)_2C_4H_4O_6$	2.45
dl Alanine	$CH_3.CH(NH_2)COOH$	2.37
dl Asparagine	$NH_2CO.CH_2CH(NH_2).COOH$	2.00
Glycine	$NH_2.CH_2.COOH$	2.00
dl Methionine	$CH_2(SCH_3)CH_2.CH(NH_2).COOH$	3.97
Urea	$NH_2.CO.NH_2$	0.80

All the above substances were laboratory grade reagents supplied by B.D.H. except Urea which was an Analar reagent.

they could get free oxygen. Incubation at 23°C lasted for eight weeks after which time the resultant mycelial mats were washed, dried at 50°C to constant weight and weighed. Their mean dry weights are shown in Table 4.

Results

The results for all the fungi were taken from a single experiment which should rule out both variation in the basal medium and the age and vigour of the inoculum. The percentages of the control were calculated and the results are shown in the form of histograms.

From Table 4 and Plates 22-29, it can be seen that C. dunensis, M. grammopodia and Peziza ammophila utilized asparagine best of all the seven sources of nitrogen supplied to them. In addition Conocybe also had good growth on ammonium nitrogen and the amino acids alanine and glycine. Growth on urea was better than that on the control whereas on nitrate and methionine growth was poorer.

The results for M. grammopodia were almost similar to those of C. dunensis but here growth on urea was almost as good as that on glycine or on ammonium nitrogen but better than on alanine.

Peziza ammophila, however, was quite different. The nitrate nitrogen was second only to asparagine. Alanine also supported some growth, glycine less; ammonium nitrogen and methionine were below control.

In Psathyrella ammophila, ammonium tartrate and urea were the only two sources to support growth. All the others either equalled or were less than that on the control, glycine and potassium nitrate in the first group,

TABLE 4

The effect of the source of nitrogen on the growth of the fungi
Incubation period 8 weeks at 23°C. Average of 2 replicates in mg.
Dried at 50°C.

Nitrogen source	Cono-cybe	Melano-leuca	Psathy-rella	Peziza	E 1	A 1	Polyporus	Panaeolus
Control	39.3	15.2	11.7	9.2	12.4	17.7	15.4	24.0
Potassium nitrate	37.7	12.2	11.5	29.2	10.7	21.7	12.2	20.0
Ammonium tartrate	139.2	21.7	18.5	6.3	19.8	42.5	58.0	43.2
dl Alanine	135.4	17.0	10.6	15.7	23.1	27.7	89.6	48.6
dl Asparagine	200.9	26.3	10.0	40.2	32.2	45.0	12.6	34.8
Glycine	141.5	21.6	11.8	10.8	37.7	57.0	40.8	-
dl Methionine	35.5	15.3	7.2	7.9	30.5	23.8	16.7	32.4
Urea	64.5	20.5	21.4	-	7.8	22.1	87.8	20.5

PLATE 22

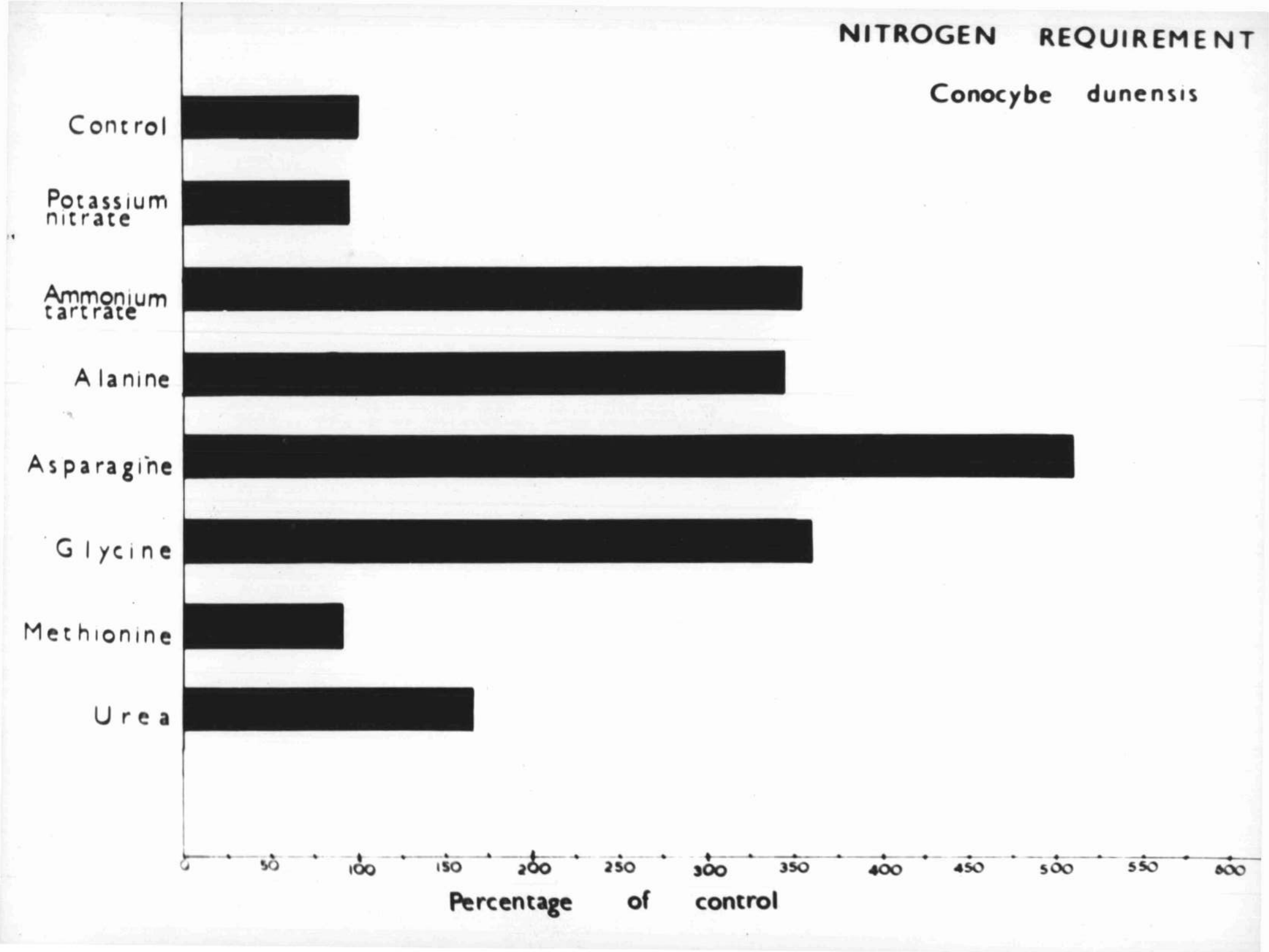

NITROGEN REQUIREMENT
Conocybe dunensis
Control
Potassium nitrate
Ammonium tartrate
Alanine
Asparagine
Glycine
Methionine
Urea
0
50
100
150
200
250
300
350
400
450
500
550
600
Percentage of control

PLATE 23

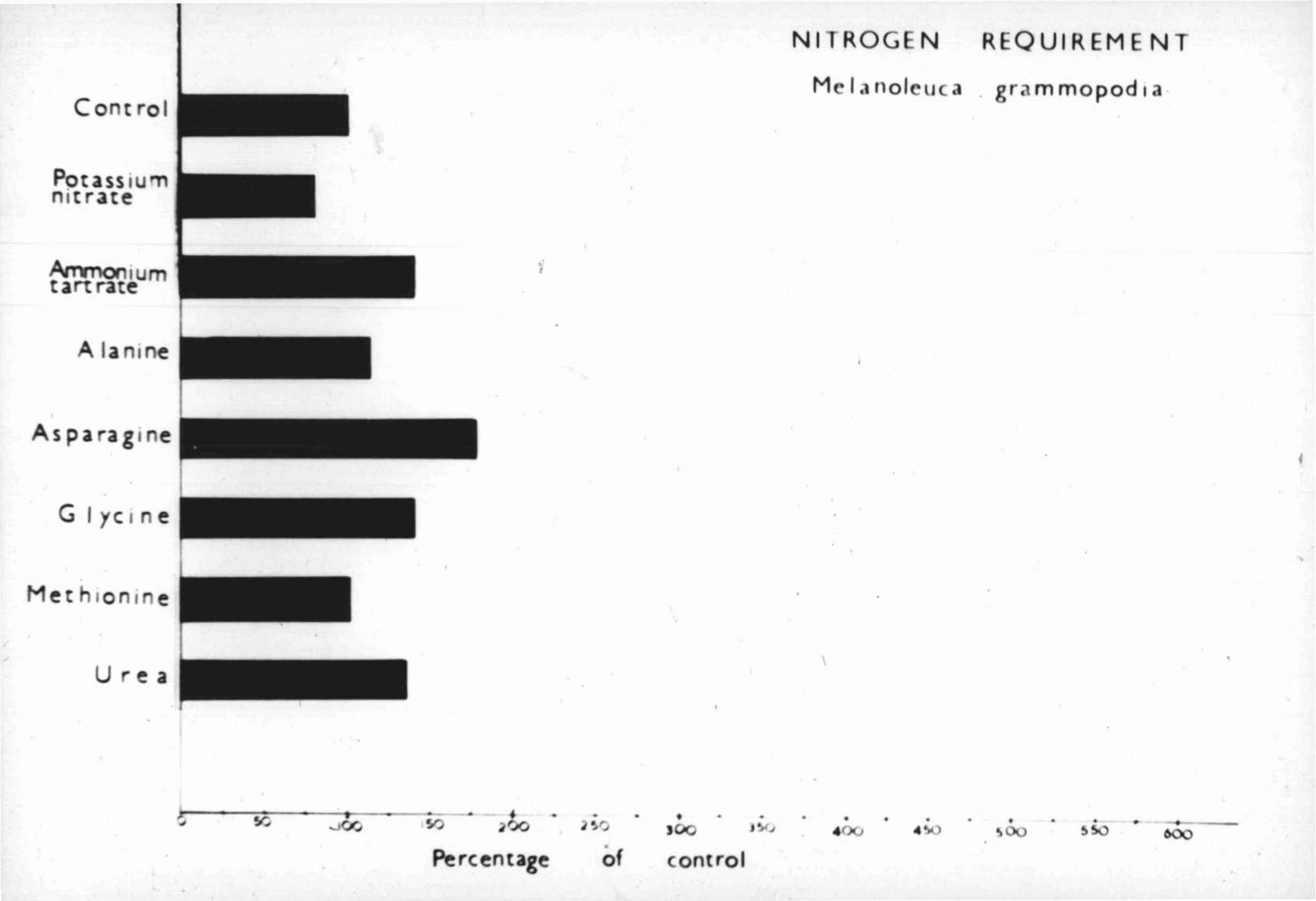

NITROGEN REQUIREMENT
Melanoleuca grammopodia
Control
Potassium nitrate
Ammonium tartrate
Alanine
Asparagine
Glycine
Methionine
Urea
0
50
100
150
200
250
300
350
400
450
500
550
600
Percentage of control

PLATE 24

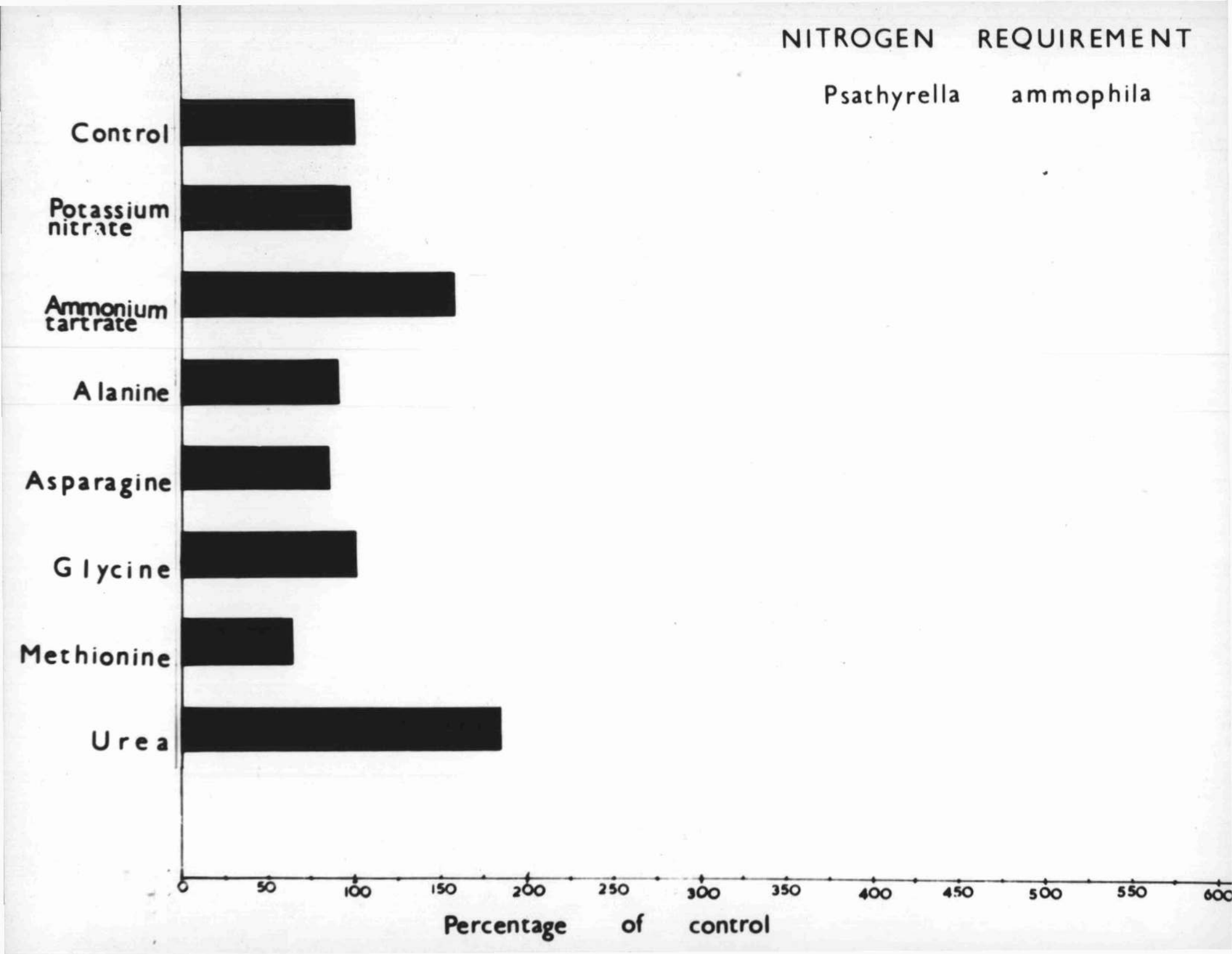

NITROGEN REQUIREMENT
Psathyrella ammophila
Control
Potassium nitrate
Ammonium tartrate
Alanine
Asparagine
Glycine
Methionine
Urea
0
50
100
150
200
250
300
350
400
450
500
550
600
Percentage of control

PLATE 25

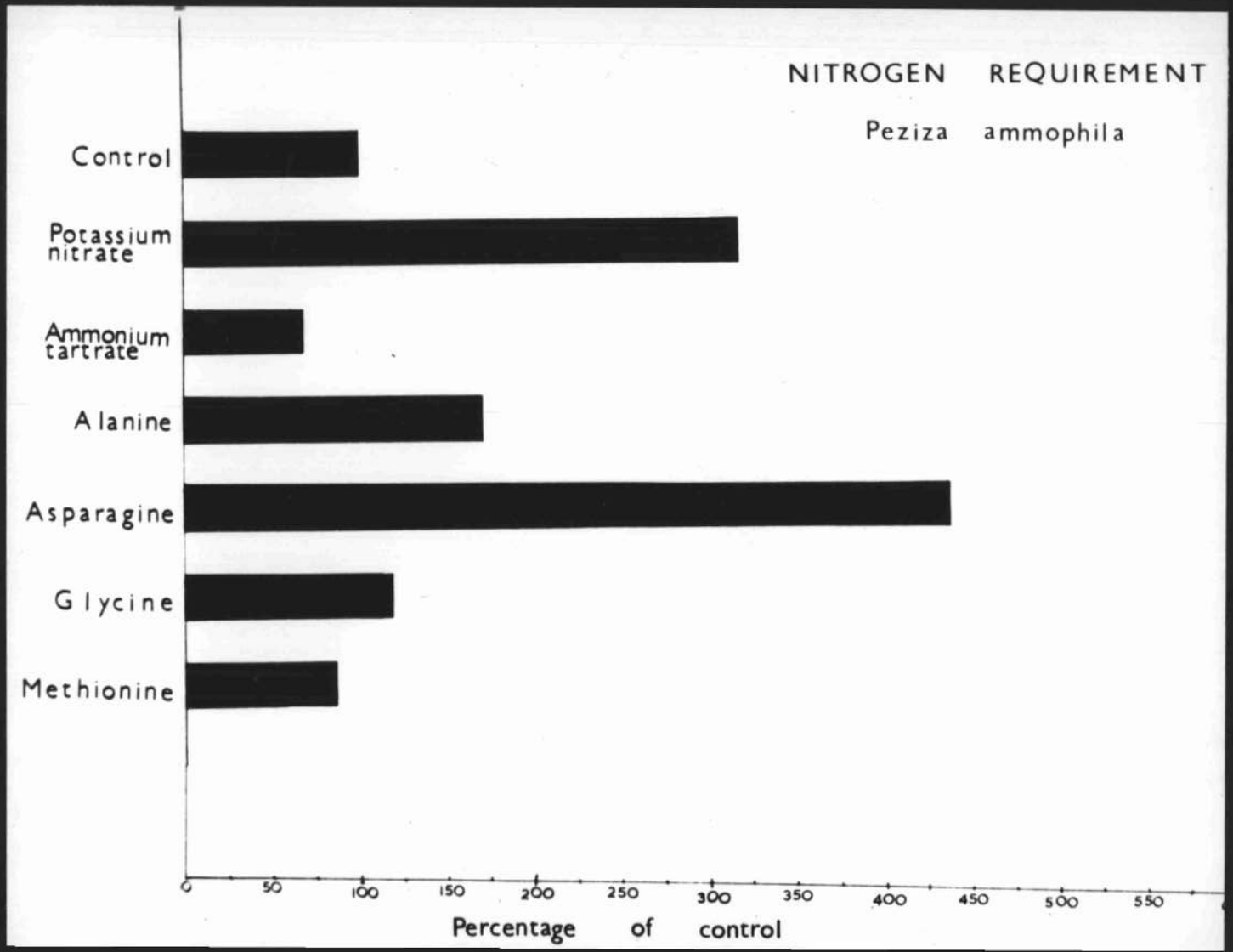

NITROGEN REQUIREMENT
Peziza ammophila
Control
Potassium nitrate
Ammonium tartrate
Alanine
Asparagine
Glycine
Methionine
0
50
100
150
200
250
300
350
400
450
500
550
Percentage of control

PLATE 26

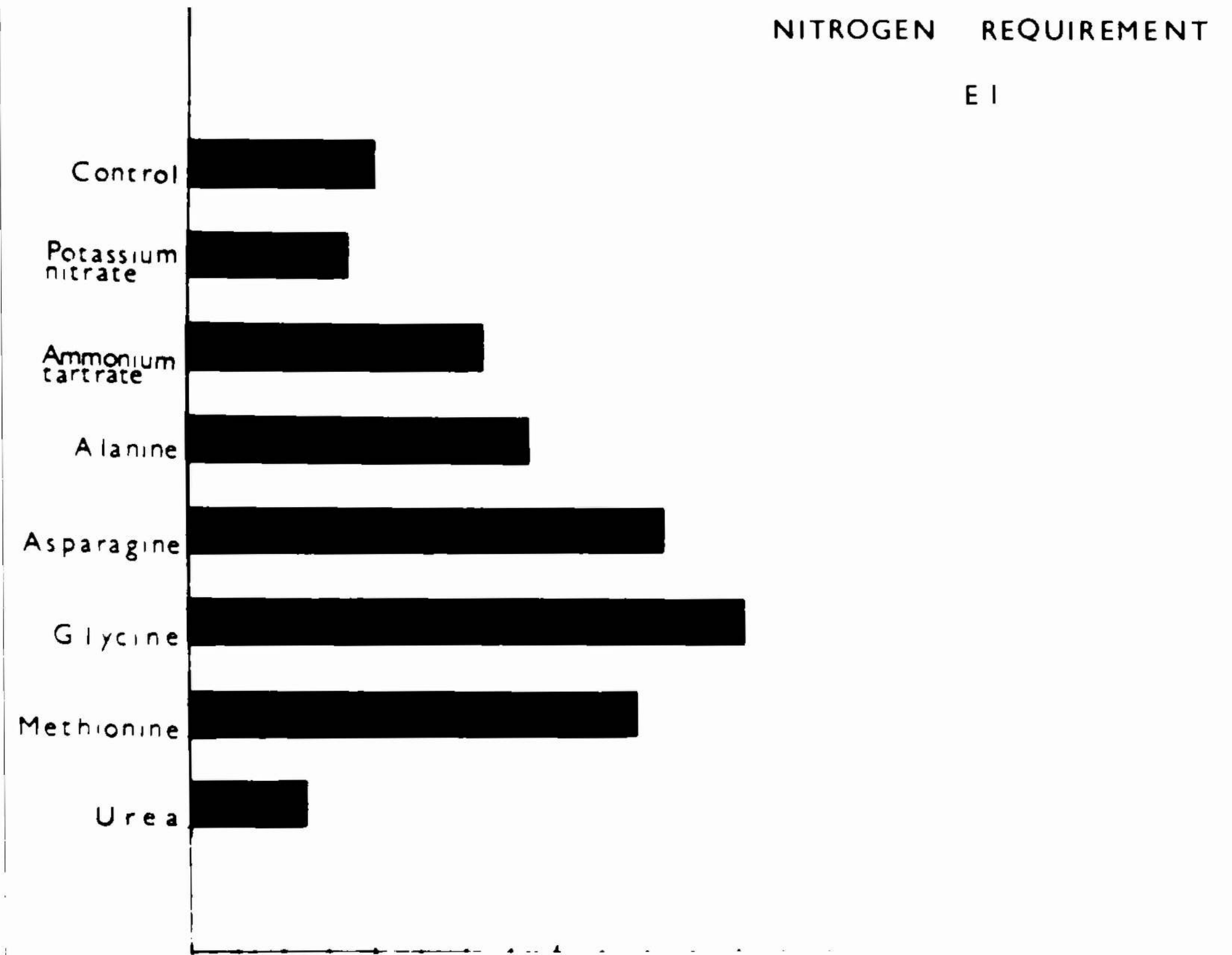

NITROGEN REQUIREMENT
E I
Control
Potassium nitrate
Ammonium tartrate
Alanine
Asparagine
Glycine
Methionine
Urea

PLATE 27

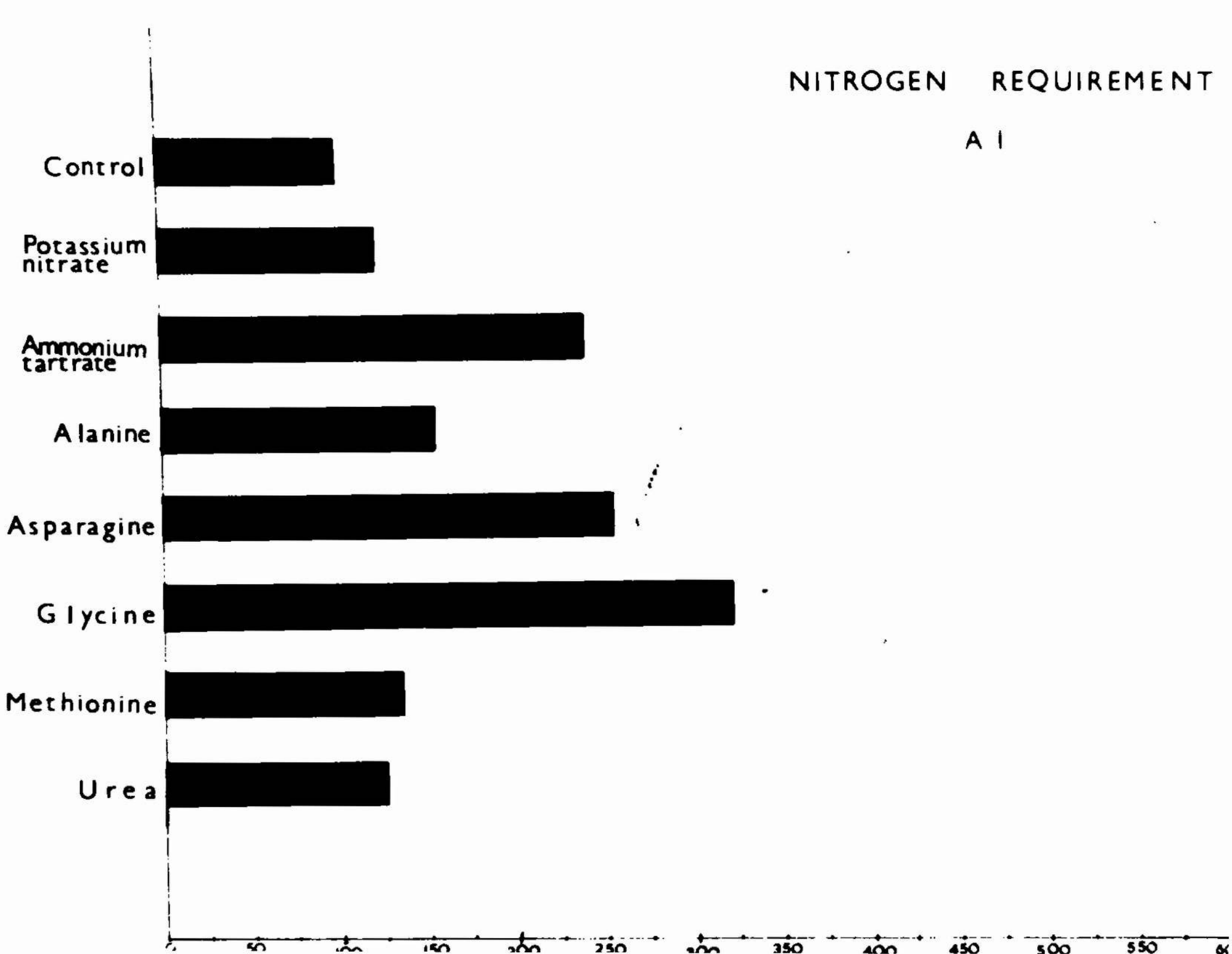

NITROGEN REQUIREMENT
A I
Control
Potassium nitrate
Ammonium tartrate
Alanine
Asparagine
Glycine
Methionine
Urea
50
150
250
350
400
450
500
550

PLATE 28

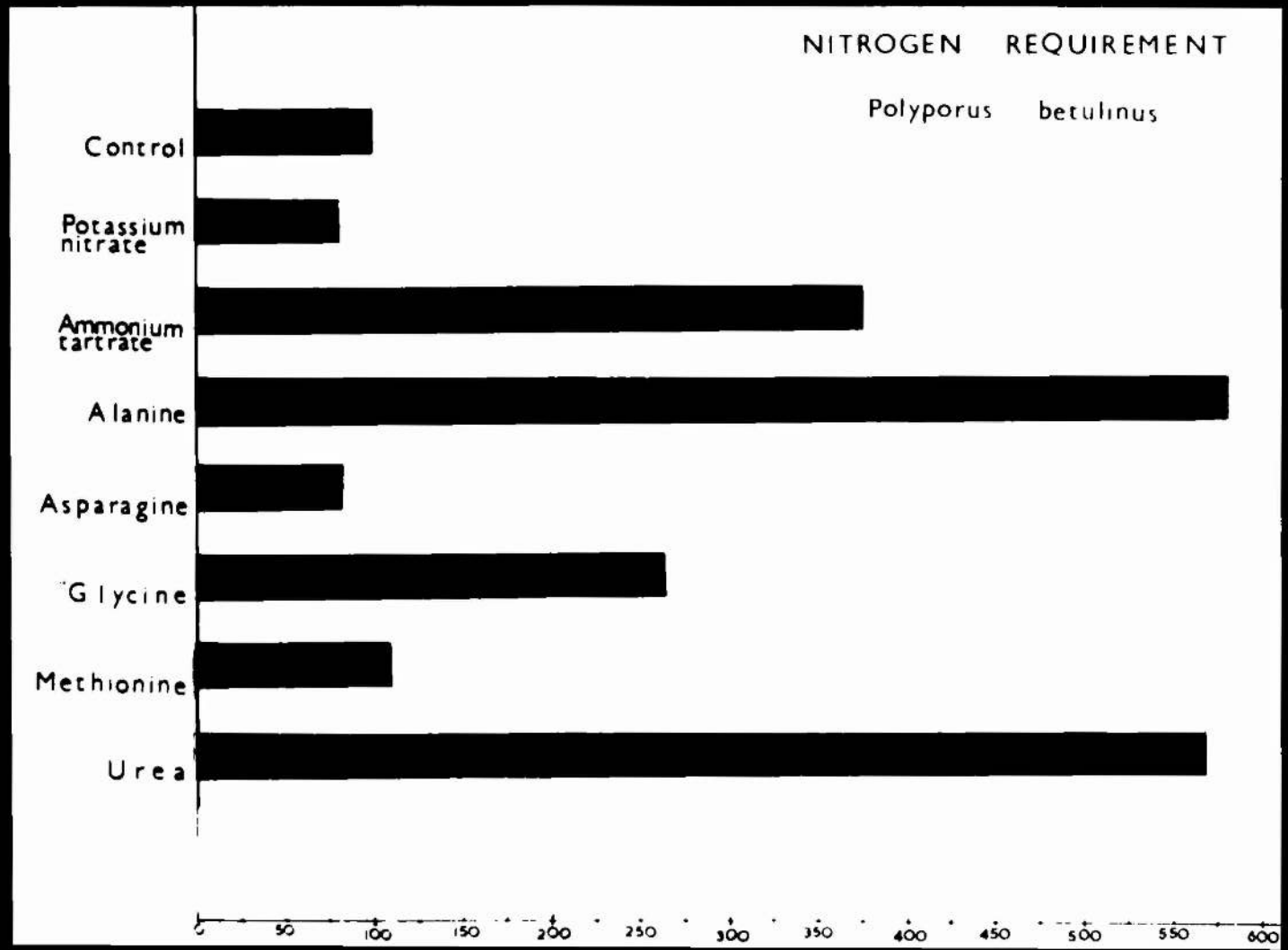
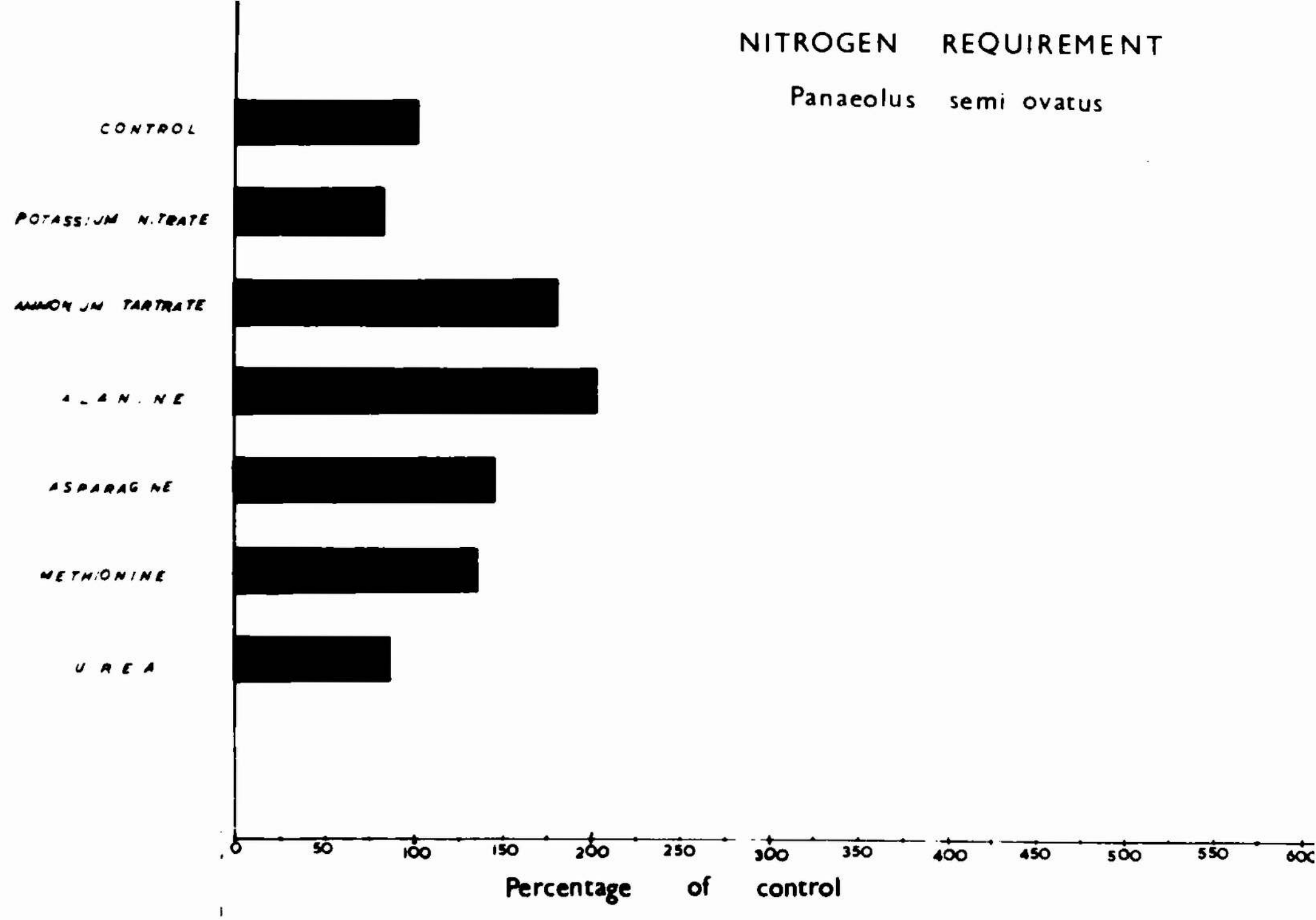

NITROGEN REQUIREMENT
Polyporus betulinus
Control
Potassium nitrate
Ammonium tartrate
Alanine
Asparagine
Glycine
Methionine
Urea
0
50
100
150
200
250
300
350
400
450
500
550
600

PLATE 29


NITROGEN REQUIREMENT
Panaeolus semi ovatus
CONTROL
POTASSIUM NITRATE
AMMONIUM TARTRATE
ALANINE
ASPARAGINE
METHIONINE
UREA
0
50
100
150
200
250
300
350
400
450
500
550
600
Percentage of control

alanine, asparagine and methionine in the second group. This suggests that this fungus is able to utilize ammonium nitrogen alone.

In the two isolates A 1 and E 1, glycine supported the best growth and asparagine the next best. In A 1, alanine and ammonium tartrate produced better growth than potassium nitrate, methionine and urea. In E 1, however, methionine was a better nitrogen source than alanine which was in turn better than ammonium tartrate. Urea and potassium nitrate did not support growth.

In the wood-rotting fungus, Polyporus betulinus, alanine supported the best growth with urea the next best. Ammonium tartrate and glycine also produced some growth. Methionine was just better than control while potassium nitrate and asparagine produced growth which were not as good as that of the control.

Discussion

Other investigators have also found that asparagine and glycine were excellent sources of nitrogen for most basidiomycetes. Fries (1955) experimentally showed that asparagine was a good nitrogen source for most of the eighteen Coprinus species studied. Most investigators have agreed that glycine and asparagine are most likely to support growth (Cochrane, 1958).

All the fungi were able to utilize organic and ammonium nitrogen. The basidiomycetes grew very slowly on nitrate nitrogen. This is in agreement with the results of other workers for basidiomycetes in general. Melin (1953) found that only a few of his investigated mycorrhiza-formers could utilize nitrates. Lindeberg (1944) found that one species of Marasmius out of

thirteen could utilize nitrates, while Norkrans (1950) found that only Tricholoma nudum of the eight species of Tricholoma studied could utilize nitrate nitrogen. Lilly and Barnett (1951) published a list of nitrate utilizing fungi but it contained only a few basidiomycetes. Garrett (1953) and Dos Santos (1963) have shown that Armillaria mellea was not able to utilize nitrate nitrogen. However, Hacskaylo, Lilly and Barnett (1954) found that among the fourteen tested wood-destroying basidiomycetes a few used nitrates very poorly. Only Polyporus distortus grew well on nitrate, though slowly. They have pointed out that slow utilization of nitrates is exaggerated in some basidiomycetes when the experiments are for short duration. This is perhaps because it is necessary for them to reduce the nitrates before utilizing them. Apparently this reduction was a slow process because when the experiments were allowed to run longer, nitrates were utilized by fungi in which this was not apparent before.

Cochrane (1958) pointed out that urea is generally recogni$_z$ed as a utilizable nitrogen source but that only experiments with cold sterilised urea are valid since urea breaks down to ammonia on autoclaving. In the current investigation urea was steam sterilized (See Sterilization in Method). The fact that all the fungi that were able to utilize urea were also able to utilize ammonium nitrogen suggests that the urea was broken down. Fries (1955) found that only two of the eighteen species of Coprinus could not utilize urea and that this was the best nitrogen source for three of them.

4c Production of Enzymes

(i) Pectinolytic enzymes

This experiment was conducted in an endeavour to discover whether the fungi were capable of digesting their way across the middle lamellae of cells. Middle lamella is known to contain a large quantity of pectin chiefly in the form of calcium pectate. A reduction in the viscosity of sodium polypectate (obtained from Sigma Chemical Co.) by a fungal extract, would indicate that the fungus had produced a pectinolytic enzyme extracellularly. This would suggest that it was capable of breaking down the pectate in the middle lamella.

Method

The experiment which is described below, is similar to that designed by Winstead and McCombs (1961) in their study of pectinolytic and cellulolytic enzyme production by Pythium aphanidermatum. In order to obtain the fungal extracts, the fungi were grown on a basal medium consisting of the following constituents:-

KH_2PO_4	3.0 grams
K_2HPO_4	2.0 "
$Mg\ SO_4 \cdot 7H_2O$	0.5 "
Asparagine	2.0 "
Distilled water	1000 ml

This medium was converted to pH6 before autoclaving.

Glucose was added to half of this medium and sodium polypectate to the other half, both at the rate of 10 grams per litre. The media were sterilized in the manner described in Section 2b(i)(b) and dispensed in 25ml quantities in previously sterilized 250ml Erlenmeyer flasks. They were then inoculated from cultures of C. dunensis, M. grammopodia, Psathyrella

ammophila, Peziza ammophila, A 1 and E 1 which were actively growing on malt extract agar for three weeks. The experiment was set up in duplicate and the flasks incubated at 23°C. After four weeks, the contents of the duplicated flasks were mixed and filtered through glass wool and gauze. The filtrate was regarded as an "enzyme extract" and was used to determine the loss of viscosity of sodium polypectate.

The "enzyme extracts" were buffered at pH 5.5 and pH 7.0 with sodium acetate/acetic acid buffer and when they were not used within one hour of filtration, they were stored in a deep freeze. A constant temperature Warburg water bath was set up at a temperature of 29.4°C (± 0.05). A Cannon-Fenske viscometer BS/IP/CF size 300 was used.

There were two enzyme extracts per fungus, (i) a "glucose enzyme extract" where the fungus was grown on the basal medium + glucose and (ii) a "pectate enzyme extract" where sodium polypectate had been added to the basal medium.

For each fungus 6 x 100ml Erlenmeyer flasks were used, each with 10ml of buffer at pH 5.5 and two at pH 7.0: one at each pH had 5ml "glucose enzyme extract" and the other had 5ml "pectate enzyme extract". The two last (of the 6) were given pH 7.0 and "glucose- or pectate-enzyme extract" and were boiled in a double boiler for thirty minutes. These served as controls.

To get an idea of the quantity of enzyme which was produced by the fungi, 0.4mg of commercial pectinase (Sigma London Chem. Co.) was dissolved in 100ml sterilized distilled water. Using 4ml of this solution which contains 0.016mg pectinase, the loss of viscosity

of 1.2% sodium polypectate was determined. The flow of water was also determined in order to calculate the viscosity losses.

All the flasks were plugged with cotton wool, the contents were homogenized by shaking and partially submerged in the water bath. After twelve hours, the liquid in each flask was poured in the viscometer and returned to the water bath for fifteen minutes for equilibration before any readings were taken. A total of six readings was taken for each mixture and an average calculated. The viscometer was washed with chromic acid, rinsed with distilled water and dried in an oven between each set of readings.

Each enzyme test was repeated three times but the results which are shown in Table 5 were taken from a single experiment. The results obtained from all the experiments were in close agreement. The results show the enzyme activity expressed as a percentage loss in viscosity calculated according to the formula used by Roboz, Barrett and Tatum (1952); Pérombelon and Hadley (1964). According to the formula, viscosity = A.

$$A = \frac{V_o - V_t}{V_o - V_s} \times 100$$

where V_o = flow time in seconds using boiled filtrate
V_t = flow time in seconds using active filtrate
V_s = flow time in seconds using water

TABLE 5

Percentage loss in viscosity of 1.2% sodium polypectate

Fungi	Grown on Glucose		Grown on Pectate	
	pH 5.5	pH 7.0	pH 5.5	pH 7.0
Conocybe dunensis	5.9	2.3	78.8	22.2
Melanoleuca grammopodia	+ 6.0	24.0	89.3	82.2
Psathyrella ammophila	3.7	+ 1.2	6.0	7.6
Peziza ammophila	3.7	10.5	9.5	10.5
A 1	6.2	13.8	23.6	23.9
E 1	11.7	0.8	92.0	91.2
Commercial pectinase	pH 5.5 91.3	pH 7.0 33.7		

Results

The ability of the fungi to produce pectinolytic enzyme(s) which is (are) capable of breaking down sodium polypectate, is shown in Table 5. It was found that all six fungi were capable of producing some amount of pectinolytic enzyme extracellularly. In all cases, the loss in viscosity of 1.2% sodium polypectate was greater when the "pectate enzyme extract" was used - a suggestion that the enzyme is adaptive to a certain extent. Loss in viscosity was better at pH 5.5 than at pH 7.0 in about 50% of the cases.

The results suggest that C. dunensis produces a large amount of pectinolytic enzyme when grown on pectate and this enzyme hydrolyses sodium polypectate at pH 5.5 to a greater extent than at pH 7.0. Even at pH 7.0 the loss in viscosity was much more than that produced

when the fungus was grown on glucose.

In M. grammopodia, + 6.0% is shown for pH 5.5 grown on glucose. This is because there was an increase in viscosity over that of the boiled control, a situation which the writer cannot explain but one that is not uncommon in viscosimetric determinations, (Strider and Winstead 1961). The "pectate enzyme extract" caused a loss in viscosity of 89.3% at pH 5.5 and slightly less (82.2%) at pH 7.0. The "glucose enzyme extract" caused a loss of as much as 24% at pH 7.0. It can, therefore, be concluded that M. grammopodia is capable of producing pectinolytic enzyme even in the absence of pectates.

In Psathyrella ammophila, the viscosity losses were always very low. Again the "glucose enzyme extract" caused an increase of 1.2% in viscosity at pH 7.0. From these results, it would appear that this fungus does not produce much pectinolytic enzyme under the conditions of the experiment.

A similar conclusion can be drawn for Peziza ammophila as for Psathyrella ammophila.

The results obtained for A 1 are also relatively low but a greater loss of viscosity was obtained with the "pectate enzyme extract". In this case the pH range over which the reaction takes place is probably wider since better results were obtained at pH 7.0.

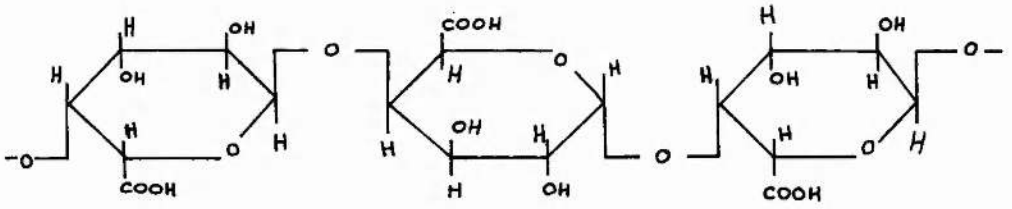
E 1 on the other hand, had very low results with "glucose enzyme extract", and very high readings with the "pectate enzyme extract". There was a slight difference in the loss of viscosity at the two pH

levels. The loss in viscosity was even greater than that caused by the commercial pectinase.

Discussion

These results are probably meaningless unless something is known about pectic substances and the enzyme systems associated with them. A brief description of these will follow.

Pectic substances are composed principally of residues of monogalacturonic acid linked through positions 1 and 4 in linear chains of various lengths. Pectic acid is the term applied to those pectic substances which are composed of colloidal polygalacturonic acid free from methyl ester groups. Pectic acid forms salts with various cations to form pectates, an example of which is sodium polypectate. When a high proportion of the carboxyl groups are esterified, the substance is known as pectin, (Kertesz 1951).



This is a diagram of polygalacturonic acid consisting of three galacturonic acid residues linked in 1:4 positions. The chain could be continued indefinitely.

The names given to the pectinolytic enzymes have been many but those used by Srivastava, Echandi and Walker (1959), will be used in this discussion.

Pectin methyl esterase catalyses the hydrolysis of the methyl ester group forming methanol and pectinic or pectic acids. It occurs chiefly in higher plants but also in some fungi, for example, a small quantity is produced by Rhizopus stolonifer, (Srivastava et al, 1959). Another pectinolytic enzyme is polygalacturonase and is known to be formed only by micro-organisms, e.g. Cladosporium cucumerinum (Strider and Winstead, 1961), Pythium aphanidermatum (Winstead and McCombs, 1961) and Pyrenochaeta terrestris (Keen and Horton, 1966). It catalyses the hydrolysis of glycosidic bonds of the pectic chain, breaking the latter to polygalacturonic acids of decreasing molecular weight, eventually forming monogalacturonic acid.

Pectic depolymerase also catalyses the hydrolysis of glycosidic bonds of the pectic chain but it differs from polygalacturonase in that the hydrolysis it causes does not proceed to the point where monogalacturonic acid is formed. It is also produced by bacteria and fungi, e.g. Rhizopus stolonifer, (Srivastava et al, 1959).

From the above description of the pectinolytic enzymes, it can be assumed that C. dunensis, M. grammopodia and E 1 were able to synthesize polygalacturonase since they caused more reduction in viscosity suggesting the smaller molecular weight of the end product, whereas the other fungi were able to synthesize pectic depolymerase or only a very small amount of polygalacturonase.

Many workers have investigated the production of pectinolytic enzymes by the lower fungi and bacteria but the literature lacks evidence that these enzymes are

produced by the higher fungi. Many of the investigations have been carried out with respect to the pathogenicity of the fungi on the higher plants. The parasitic attack of plant tissues by facultative microorganisms has often been associated with the ability of the pathogen to produce pectic enzymes.

Husain and Rich (1958) studied the extracellular pectolytic enzymes of Cladosporium cucumerinum, an important pathogen causing the scab of cucumber. They found that enzyme action is an important part of the pathogenic processes of this disease. They thought that the cells were killed in advance of actual invasion of the pathogen probably because the cellulose of the cell walls in susceptible tissue was altered. They detected the production of polygalacturonase in their viscosimetric experiments and believed that the disintegration of cucumber tissues was partly caused by this enzyme produced by the pathogen.

Srivastava, Echandi and Walker (1959), found that Rhizopus stolonifer, the cause of soft rot of sweet potato, produced small amounts of polygalacturonase, pectic depolymerase and very little pectin methylesterase, in culture media but large quantities of all three on raw sweet potatoes. Further studies were carried out by Srivastava and Walker (1959) when they developed the "well method" which they used to study the mechanism of infection of sweet potato by Rhizopus stolonifer. The "well method" (making a well in a healthy potato and using that as the site of inoculation) was developed to verify the statement of Harter, Weimer and Adams (Srivastava and Walker 1959) that the pathogen

penetrates primarily if not entirely through wounds. Srivastava and Walker found that infective hyphae were capable of parasitic activity but not the germinating spores and concluded that the pectolytic enzymes alone were not responsible for the parasitism of the fungus since an addition of the enzyme to the spore suspension did not cause rapid infection. They thought that some other metabolite might be also responsible.

Winstead and McCombs (1961), reported that Pythium aphanidermatum, which incites a cottony-leak disease of cucumber, produced depolymerase and endopolygalacturonase in culture filtrates and these degraded sodium polypectate into smaller fragments. They found that there was greater reduction in viscosity by polygalacturonase when the fungus was grown on glucose than on sodium polypectate.

A high correlation between pathogenicity and production of pectic enzymes in vitro was demonstrated by Barker and Walker (1962) on strains of Pellicularia filamentosa. Leal and Villanueva (1962) found high pectolytic activity with all the pathogenic strains of Verticillium species whereas no activity was detected in the non-pathogenic strains.

Keen and Horton (1966), reported that there was a high production of polygalacturonase from onion roots infected by Pyrenochaeta terrestris than from non-infected roots. They stated that the synthesis of polygalacturonase during pathogenesis suggests involvement of the enzyme in fungal ramification of host tissues.

On the other hand, Pérombelon and Hadley (1964), produced results showing that pathogenic and symbiotic strains of Rhizoctonia solani and also typical orchid symbionts were capable of producing a variety of pectic enzymes in quantities unrelated to their symbiotic or parasitic character. They, therefore, concluded that in mycorrhiza host-endophyte systems, pectic enzymes may well be of secondary importance and may be synthesized by all types of endophytes only as and when required e.g. for the passage of hyphae through cell walls.

It may be expected that the nature of the chemical and physical association of galacturonides and non-galacturonides (e.g. cellulose) of the cell wall will determine the extent to which pectic substances undergo enzymic degradation in plant tissues. The nature of the association will vary from species to species, from one tissue to another and within the same tissue depending upon its nutritional status and its age, (Elarosi 1958). Edgington, Corden and Dimond (1961) stated that calcium deficient tomato plants were more susceptible to Fusarium wilt than normal plants and plants which were treated with a-naphthalene acetic acid which induces calcium bonding. Since the middle lamellae are made up of calcium pectate, then in calcium deficient plants the pectic material is subject to hydrolysis by pectic enzymes of the pathogen. Treatment with calcium and growth regulator may alter host resistance to the pathogen by changing the character of the host pectic substances in such a manner as to make them resistant to hydrolysis by the pathogen.

The ability to elaborate pectic enzymes is widely distributed among organisms, since many micro-organisms must deal with pectic substances in their metabolism (Bateman and Miller, 1966). Their ability to produce pectic enzymes, therefore, does not indicate that they possess pathogenic potentialities. It is significant that many pathogenic fungi are capable of synthesizing pectic enzymes (Husain and Rich, 1958; Barker and Walker, 1962; and Leal and Villanueva, 1962), but this is only one of the factors involved in pathogenesis. Where direct penetration of the host is involved, it is possible that pectolytic enzymes may be involved together with others e.g. cellulase, in effecting the breaking of the host cell wall. Since the enzyme causes maceration of host tissues it might indirectly facilitate the invasion of the tissues by a pathogen. In most of the cases cited above, the pectinolytic enzyme was reported for organisms grown in culture. This would suggest that the enzymes may be more important in the saprophytic phase of the development of some plant pathogens (Hancock and Millar, 1965).

In light of the above observations, it can be assumed that since the fungi on which the present experiment was carried out are capable of synthesizing pectinolytic enzymes, it is possible that they possess pathogenic potentialities. On the other hand, their ability to produce pectinolytic enzymes in vivo was not investigated. Consequently, there is no evidence

that they do so in nature. These enzymes may be playing a much more important role in their saprophytic life (Hancock and Millar, 1965) and they may be synthesized if and when necessary by the fungi, e.g. when they invade new substrates as they grow through the soil or through living or dead plant tissues.

4c (ii) Cellulolytic Enzymes

Method

The cellulolytic properties of the fungi were determined qualitatively by growing them on solidified nutrient agar with (i), ball-milled cellulose + starter glucose and (ii), carboxymethylcellulose + starter glucose. Positive results were obtained in some cases.

It is a well established practice for fungi to be qualitatively tested for their ability to break down cellulose on agar plates containing a medium with precipitated cellulose. The production of extracellular cellulolytic enzymes should transform the insoluble cellulose into soluble sugars producing clear zones around the fungal colonies. The method employed in the current work is the same as that used by Norkrans (1950) with which she determined the cellulolytic properties of different Tricholoma species. The following medium was used:-

Cellulose *	5.0 grams
$NH_4H_2PO_4$	2.0 "
KH_2PO_4	0.6 "
K_2HPO_4	0.4 "
$MgSO_4 \cdot 7H_2O$	0.5 "
Ferric citrate	5.0 mgs
$ZnSO_4 \cdot 7H_2O$	4.4 "
$MnSO_4 \cdot 4H_2O$	5.0 "
$CaCl_2$	55.2 "
Difco agar	15.0 grams
Glucose	0.75 "
Distilled water	1000 ml

*or carboxymethylcellulose which did not go into solution but produced an opaque medium.

Norkrans (1950) used either chopped and washed filter paper, precipitated cellulose or cellulose combined with different amounts of glucose (10 or 50 mg per dish). Her experiment ran for 45 days and she found that the amount of cellulose decomposed was inversely proportional to the amount of glucose added to the medium.

In the present investigation, ballmilled cellulose (Whatman's standard grade cellulose powder) and carboxymethylcellulose, both sold for chromatography purposes, were used as the major carbon source. Glucose was added with the hope that it would be just sufficient to enable the fungi to start growing.

The solutions were sterilized by autoclaving, (see Section 2b(i)(b). While pouring in plastic petri plates, the flasks were constantly shaken to facilitate equal distribution of the cellulose particles. The inoculated plates were incubated at 23°C and examined every three or four days.

The fungi used were C. dunensis, Psathyrella ammophila, M. grammopodia, Peziza ammophila, Panaeolus semi-ovatus, E 1, A 1 and Tricholoma nudum, a known cellulose decomposer, (Norkrans, 1950) which was used as a test fungus.

The layer of agar in the petri dishes had to be thin to enable even small quantities of decomposition to be easily detected. At the same time majority of the fungi grew very slowly and in a short time the agar dried out if the layer was too thin. Furthermore, all native cellulose, precipitated cellulose, filter paper, wood cellulose and dewaxed cotton require a long time for decomposition, (Norkrans, 1950). It was, therefore,

very difficult to detect clearing of the media and small quantities could have been overlooked. Therefore, the plates were examined microscopically and a marked difference between the concentration of cellulose fibres under and around the colonies was used as a positive result.

Results

The results of this experiment are shown in Plates 30-40. None of the fungi which were used in this investigation was able to break down ball-milled cellulose up to the close of the experiment which lasted for eight weeks. Five of them, P. semi-ovatus, T. mudum, A 1, E 1 and C. dunensis, decomposed carboxymethylcellulose - a cellulose derivative.

At the end of two weeks, P. semi-ovatus had developed profuse growth on the agar and clear zones were present around the fungal growth. At this time no other fungus was showing such positive results for cellulose decomposition.

At the end of three weeks, T. mudum had started to break down the cellulose fibres in the carboxymethylcellulose but not enough to cause distinctly clear zones. By the end of four weeks, the mycelium had covered the agar surface and evidence of clearing was obvious under a microscope at x10 magnification. Plate 31 shows that most of the fibres had disappeared by then.

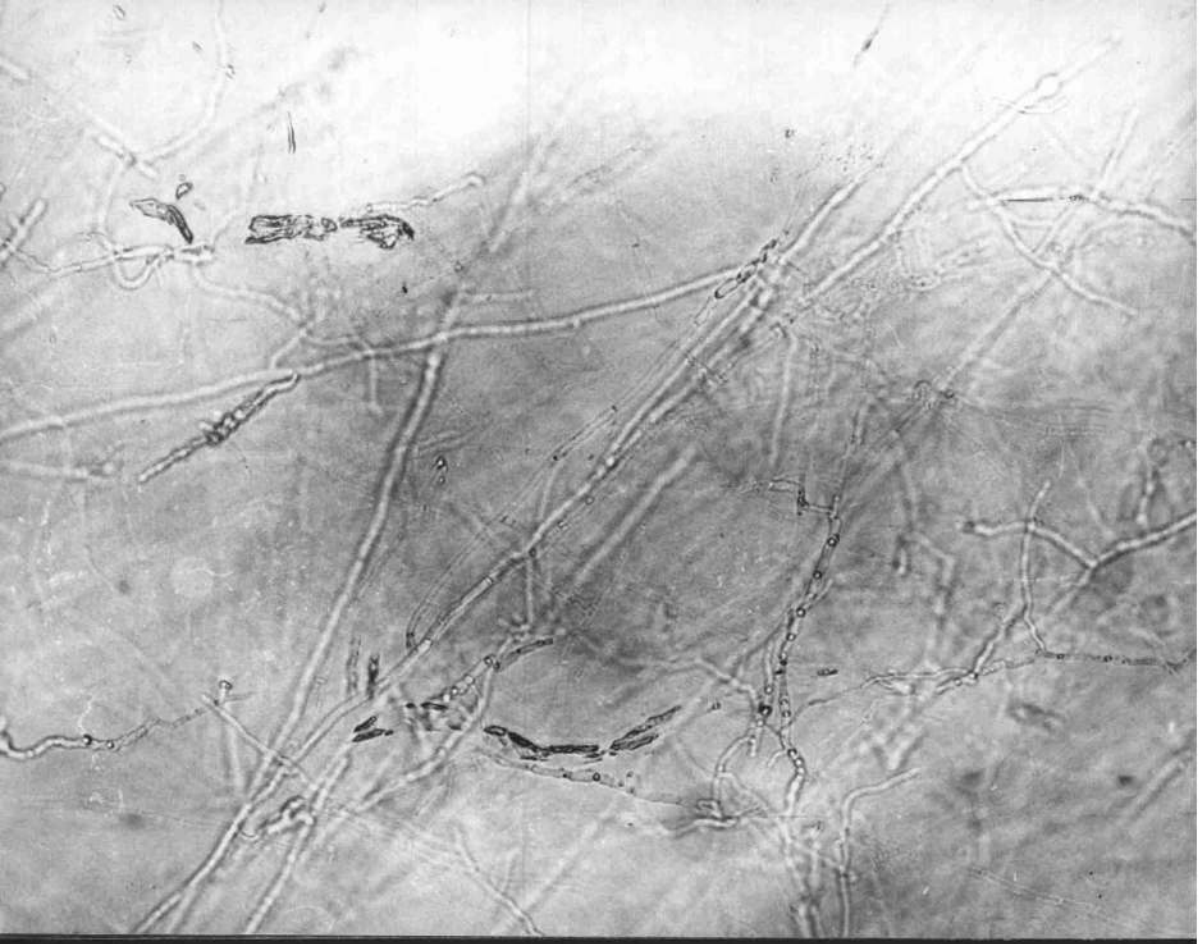
A 1 showed signs of decomposition after three weeks (Plate 39) and by the end of five weeks most of the fibres in the vicinity of the older hyphae had been

PLATE 30

Decomposition of carboxymethylcellulose (CMC) by Panaeolus semi-ovatus. Arrow points to fragments of CMC left undecomposed after two weeks.

PLATE 31

Tricholoma nudum growing on CMC for three weeks. Most of the fibres have been decomposed.



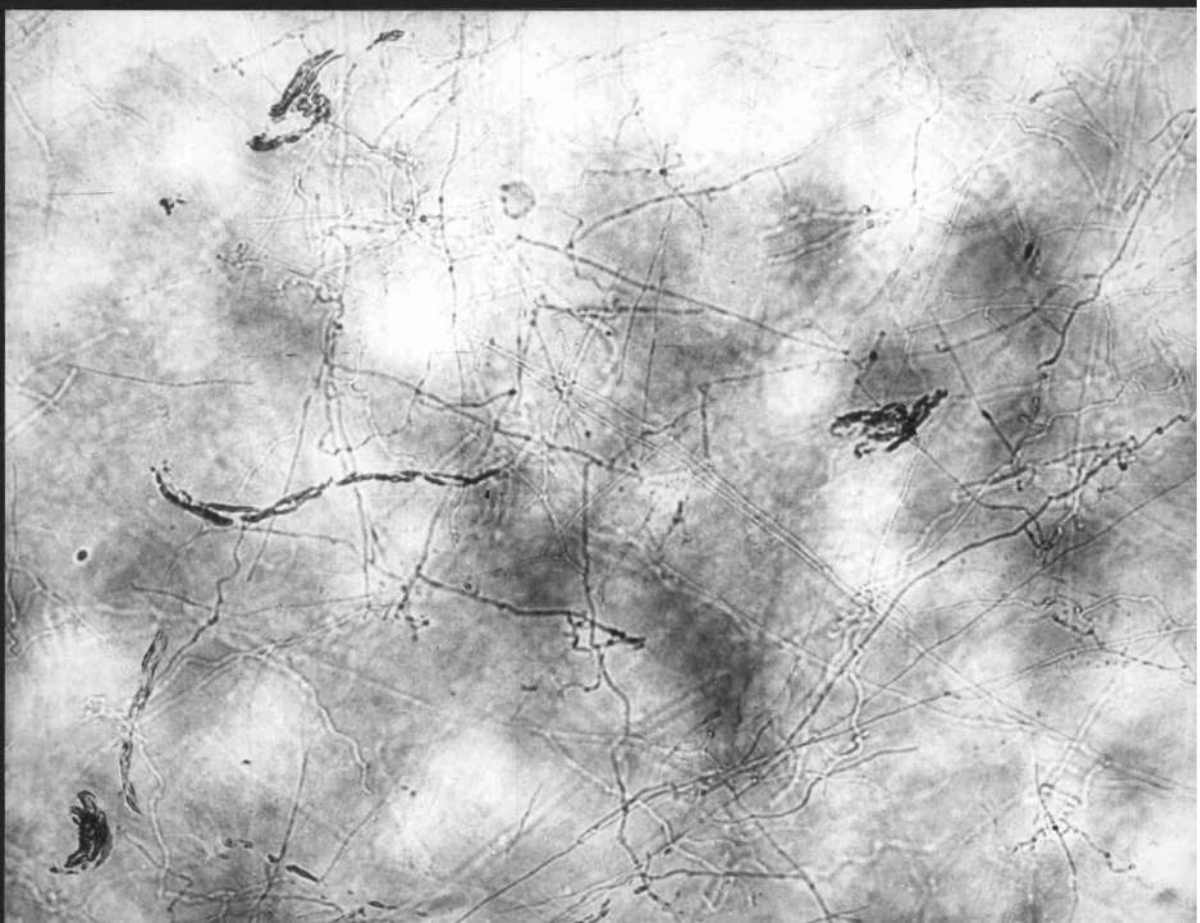


PLATE 32

C. dunensis on carboxymethylcellulose for six weeks. No decomposition in the region of the hyphal tips.

PLATE 33

Cellulase activity in the older region of *C. dunensis*.

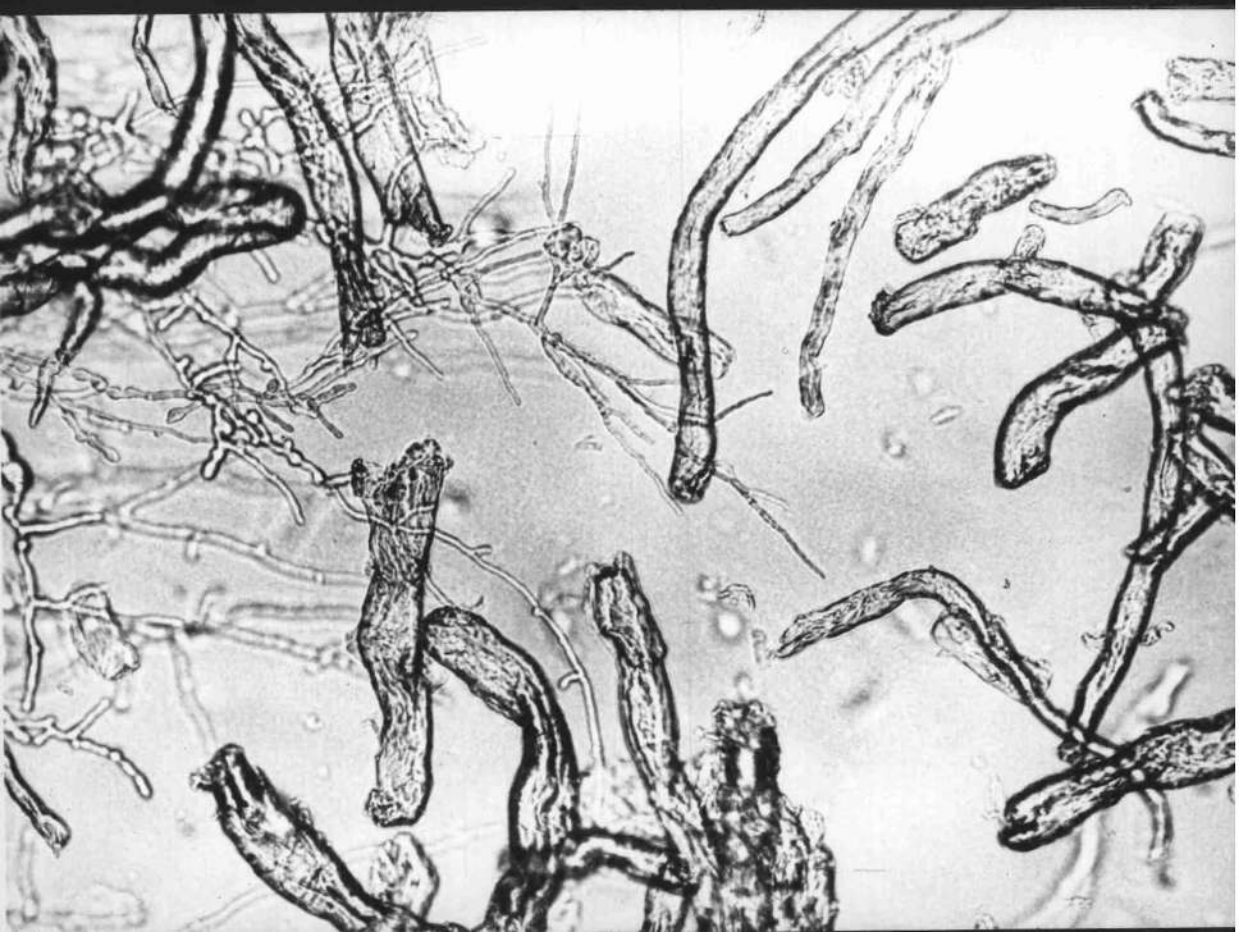


PLATE 34

M. grammopodia growing on carboxymethylcellulose for six weeks. No decomposition of the fibres.

PLATE 35

Psathyrella ammophila growing on carboxymethylcellulose for six weeks. No degradation of the fibres.

PLATE 36

Peziza ammophila growing on carboxymethylcellulose for six weeks. No degradation of the fibres.

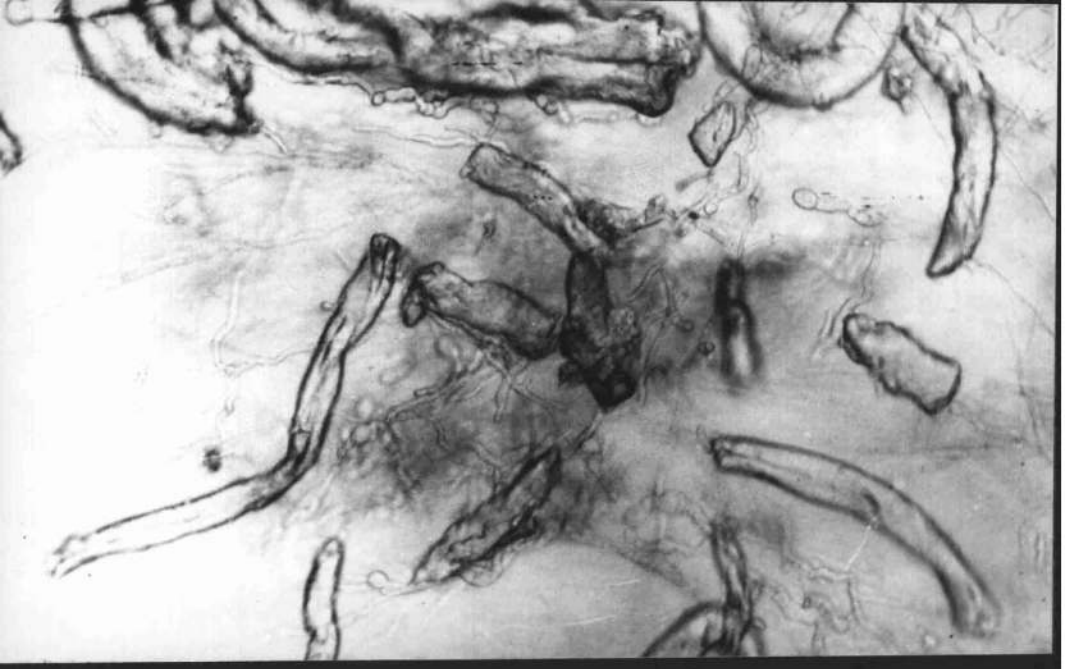


PLATE 37

E 1 growing on carboxymethylcellulose for six weeks. No degradation in the young regions.

PLATE 38

E 1 growing on carboxymethylcellulose for six weeks. The fibres are being decomposed in the older regions.

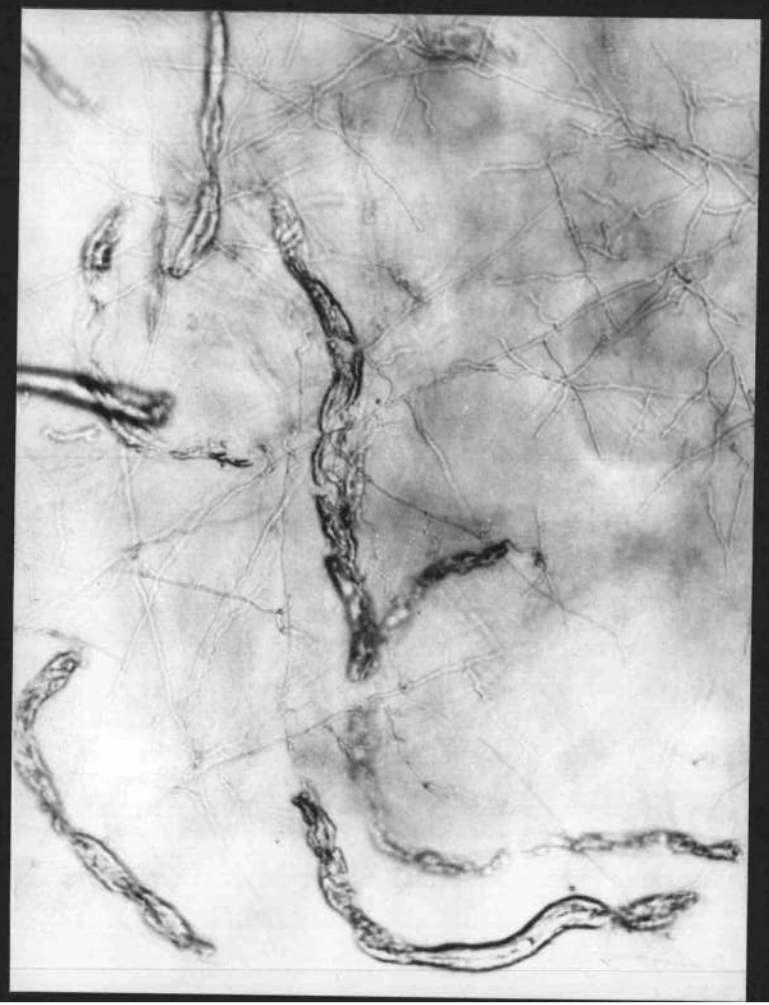
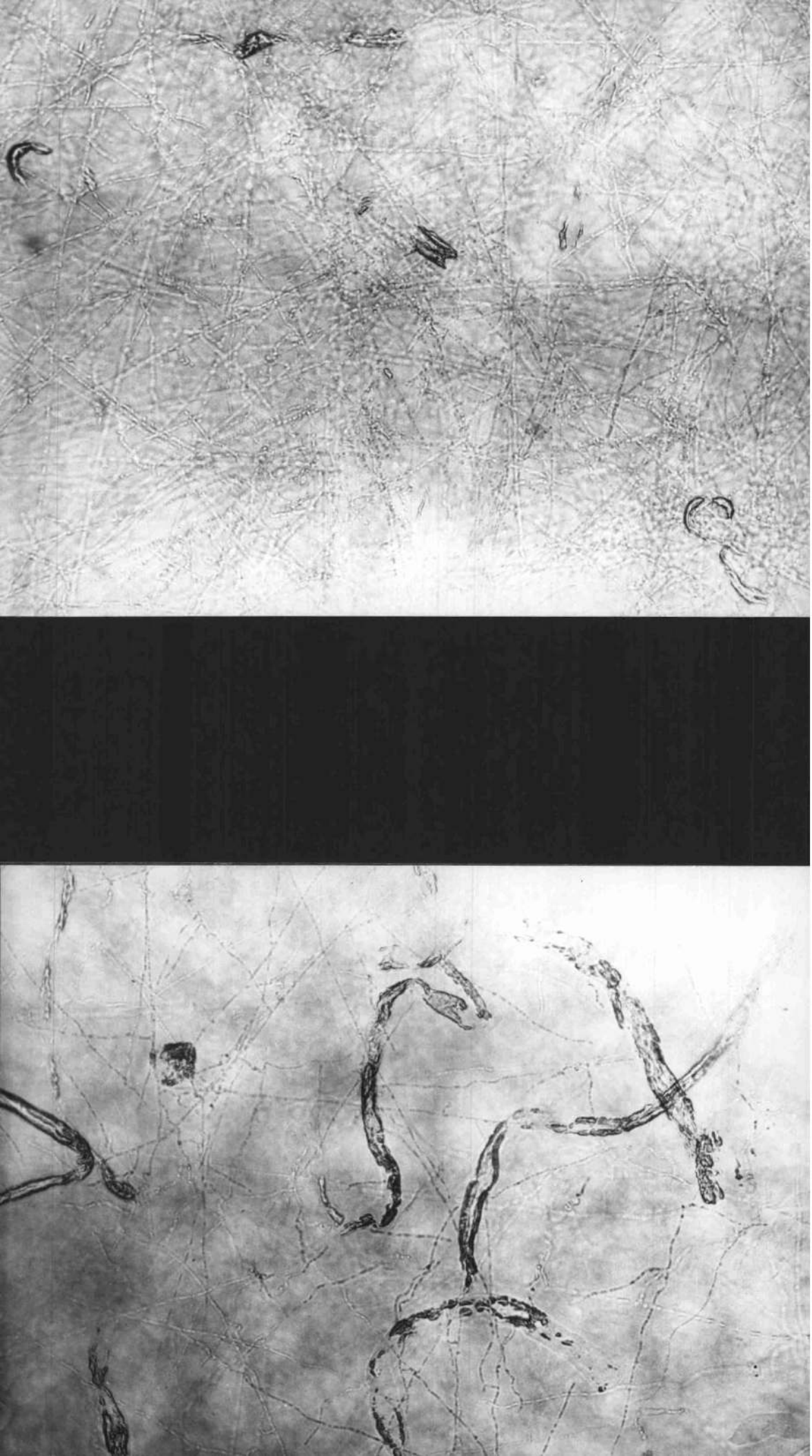


PLATE 39

A 1 growing on carboxymethylcellulose for three weeks. Fibres undergoing decomposition.

PLATE 40

A 1 growing on carboxymethylcellulose for five weeks. Most of the fibres have been decomposed.



decomposed, (Plate 40). The enzyme seems to be adaptive and is not produced until the fungus is well established.

In C. dunensis and E 1, the rate of decomposition was even slower than the others and clearing was observed after six weeks and even then the amount of cellulase activity was relatively small (Plates, 32, 33, 37 and 38). This suggests that the enzyme is definitely adaptive or that the amount that was produced by these fungi was negligible.

Plates 34, 35 and 36 show that there was no degradation of the cellulose fibres with M. grammopodia, Psathyrella ammophila and Peziza ammophila.

Discussion

Some fungi have been found to be able to produce enzymes in culture which can hydrolyse soluble cellulose derivatives, e.g. Rhizoctonia solani, (Bateman, 1964). These cellulases which are produced in culture are called C_x. Other fungi are able to produce enzymes which can hydrolyse derived cellulose and native cellulose, e.g. Fusarium oxysporum, (Husain and Dimond, 1960). The enzyme which degrades native cellulose has been given the name, C_1.

It would, therefore, appear that none of the fungi which were used in the current investigation was able to produce C_1 and only C. dunensis, E 1, A 1, P. semi-ovatus and T. mudum could produce C_x.

As in the case of pectinolytic enzymes, many investigations relate the production of cellulose decomposing enzymes with pathogenicity.

Husain and Dimond (1960), found that Fusarium oxysporum was capable of degrading native cellulose and derived cellulose. Cellulase production was found to be responsible for wilting in tomato cuttings. They postulated that the hydrolytic products of cellulase activity provided Fusarium oxysporum with the carbohydrates necessary for its continued development in the host. Cellulase activity may also be involved in the escape of the pathogen from the vascular tissue in the advanced stages of the disease when the host is in a dying condition. In this condition the host tissue will be depleted of sugars; cellulase and pectinolytic enzymes might be produced in large quantities and could rapidly disintegrate the host dead tissues thus facilitating the escape of the pathogen.

Winstead and McCombs (1963), pointed out that the production of symptom of anthractnose on cucumber - collapse of tissues in the foliage and in the fruit, by Glomerella cingulata var. orbiculare, G. manana and G. lagenaria, suggests that pectinolytic and cellulolytic enzymes were in operation. When these fungi were grown on carboxymethylcellulose and cucumber slices, cellulase was always present in the filtrates. The viscosity loss of carboxymethylcellulose was always highest when extracts from the cucumber slices were used.

Barker and Walker (1962), did not associate the production of cellulase by Rhizoctonia solani with pathogenicity. Though the fungus produced enzymes capable of hydrolysing carboxymethylcellulose, they said that this is different from the ability to attack cell wall. To attack native cellulose appeared to be of much greater significance in pathogenesis and saprogenesis.

Two years later, Bateman (1964), investigated the effect of R. solani on the cellulose in the cell walls of infected tissues. He found that the cell walls on the older portions of lesions lost their birefringent properties and interpreted this as evidence of destruction of the crystalline cellulose in the host by the C_1 enzyme of the fungus. Bateman concluded that the cellulolytic factors associated with the above symptoms were of secondary importance with regard to pathenogenesis or the initiation of the disease, or both, and that these factors may be more closely associated with the saprophytic activities of the pathogen in previously killed tissues.

Spaldings (1963), found that Rhizopus stolonifer produced cellulolytic enzymes in vivo and in vitro. This fungus caused the decay of strawberries, peaches and sweet potatoes during storage. It produced cellulolytic enzymes during the decay of the sweet potatoes.

Both white rot and brown rot fungi synthesize cellulase and are capable of breaking down native cellulose (Johansson, 1966). The ability to utilize cellulose was regarded by Melin (1948) as an essential for saprophytic fungi. Chesters (1960) suggested that a demonstration of cellulolytic ability in pure culture, (and isolation of particular fungi from litter at various stages of decay), must at least prove the potential for competitive saprophytic survival in litter. Garrett (1963) found that an abundant supply of soluble nitrogen in the soil promoted an increase in the survival of four of the five fungi on which he experimented. He found a positive effect of nitrogen on the

saprophytic survival of a pathogen in dead infected host tissues. The areas of enzymic activity around individual hyphae in colonized substrates eventually become exhausted and the mycelium dies from carbohydrate starvation unless there is an adequate supply of soluble nitrogen. This would permit the formation of new young hyphae that can explore and decompose fresh regions of the substrates. In his opinion a slow rate of cellulose decomposition is an economical rate of substrate utilization and tends to conserve substrate reserves resulting in a longer period of saprophytic survival.

Therefore, the production of cellulase has been linked both with pathogenicity and saprogenicity and the ability of a fungus to synthesize this enzyme does not make it a potential pathogen any more than a potential saprophyte. Norkrans (1963) stated that in fully mature woody tissues the α-cellulose amounted to 40-60% of the oven dry material but in non-woody tissues e.g. in straw and grasses the cellulose accounted for only 12% of the oven dry weight.

The fungi which live on the sand-dunes, where the amount of cellulose is so low could not rely entirely on this for their supply of carbon source. However, the ability to synthesize cellulase could be very advantageous to them both for their saprophytic and/or their pathogenic survival. E 1 and A 1 which were isolated from the roots of Elymus and Ammophila respectively, would be able to break down the cellulose cell walls of the cortical cells of their host and invade new tissues. In fact they are able to do so as can be

seen from Plates 12 and 59 where the fungi apparently digested the cell walls of the cortical cells and now occupy the lysigenous cavity so formed. Conocybe is also potentially capable of digesting the native cellulose of cell walls. The others, that is, Psathyrella, Peziza and Melanoleuca which did not break down cellulose in culture might be able to do so in nature where the conditions are different e.g. the presence of other micro-organisms, pH, temperature and nutrients.

4c (iii) Production of Polyphenol Oxidases

In 1938 Davidson, Campbell and Blaisdell investigated 210 species of wood decaying fungi and confirmed Bavendamm's generalization that, when tested on media containing gallic and tannic acid, some fungi formed a dark diffusion zone in the medium adjacent to the fungus mycelium. According to these workers, the mycelia of these fungi produced an extracellular enzyme which is called polyphenol oxidase or laccase. This enzyme catalyses the oxidation of the polyphenols in the tannic and gallic acids giving rise to products containing dark coloured quinones. The fungi which are able to produce this enzyme are called white rot fungi and they are able to decompose both the lignin and the cellulose of wood. Brown rot fungi, on the other hand, decomposed only the cellulose of wood and generally had no oxidizing action on the polyphenols.

Mildred Nobles (1958) thought Bavendamm's technique was good but laborious. She devised a more rapid test in which a drop of freshly prepared alcoholic solution of gum guaiac was placed on the growing mycelium in the petri plates. A characteristic blue colour which is produced immediately indicates the presence of the oxidase production. (This method was not used because the gum guaiac was not obtainable when it was ordered. The reply came that it was outdated and not kept in stock any more.)

In the present experiment, therefore, the old method of Bavendamm (Davidson et al, 1938), was used to investigate the production of polyphenol oxidase by C. dunensis, M. grammopodia, Psathyrella ammophila, Peziza ammophila,

E 1, A 1, Polyporus betulinus and Polyporus abietinus. The last two were used as test fungi - the isolate of P. betulinus used giving a negative reaction and P. abietinus a strong positive reaction on the gallic and tannic acid media (Davidson et al, 1938).

The acid media were made up as follows:- 20 grams Difco agar and 15 grams malt extract were dissolved in 850ml distilled water in an Erlenmeyer flask. In another flask there were 150ml distilled water. The flasks were autoclaved at 15 lbs. pressure for fifteen minutes. After removal from the autoclave, 5.0 grams of tannic acid were dissolved in the flask with the sterilized water and this was added to the agar when the agar was cool. (Heating gallic or tannic acid with agar causes hydrolysis of the agar.) The same procedure was repeated but gallic acid was used instead of tannic acid. Tannic acid caused the medium to become milky but gallic acid did not affect the colour. The media were then poured in plastic petri plates and inoculations were made under aseptic conditions. The inoculations were made from cultures which had been growing on 2% malt extract agar for three weeks. The inoculants were put upside down and gently pressed down on to the agar. This was to allow all the hyphae to be in direct contact with the medium. They were incubated at 23°C.

Results

After five days of incubation, the photographs which are shown in Plates 41-48 were taken. As can be seen from these plates, all the fungi except

P. betulinus and Peziza were able to produce coloration of the agar indicating their ability to synthesise polyphenol oxidase.

In the case of Polyporus betulinus, there was no discoloration under or around the mat of hyphae. The growth which was produced on the gallic acid agar was better than that on the tannic acid medium.

With Peziza ammophila the inoculants retained their original colour both on the tannic acid and the gallic acid media but there was no growth even after two weeks of incubation.

Polyporus abietinus showed positive reaction - the dark brown diffusion zone was very intense and opaque. There was no growth.

In Psathyrella ammophila and Conocybe dunensis, a wider diffusion zone was produced on gallic acid than on tannic acid. In both cases there was no growth and the intensity of discoloration on the gallic acid medium was comparable to that of P. abietinus. On tannic acid agar the diffusion zone was less intense especially in the case of C. dunensis.

In M. grammopodia, the diffusion zone was also dark brown in colour but extended a short distance from the edge of the inoculum. The intensity was similar on both media.

In E 1 and A 1, the inocula were dark brown in colour but the diffusion zone was only light brown and extended only a short distance from the inocula.

PLATE 41

Polyporus betulinus grown on tannic acid (right) and gallic acid (left) for 5 days. Negative reaction.

PLATE 42

Polyporus abietinus producing positive reaction on tannic acid (right) and gallic acid (left). There was no growth after 5 days.

PLATE 43

C. dunensis grown on tannic acid (right) and gallic acid (left) for 5 days. Positive reaction was greater on the gallic acid medium. No growth.

D

C
C

PLATE 44

M. grammopodia producing no growth but positive reaction on tannic acid (right) and gallic acid (left) after 5 days.

PLATE 45

Psathyrella ammophila on tannic acid (right) and gallic acid (left) for 5 days. There was no growth but a positive reaction.

PLATE 46

Peziza ammophila showing no growth and a negative reaction on tannic acid (right) and gallic acid (left).

T
T

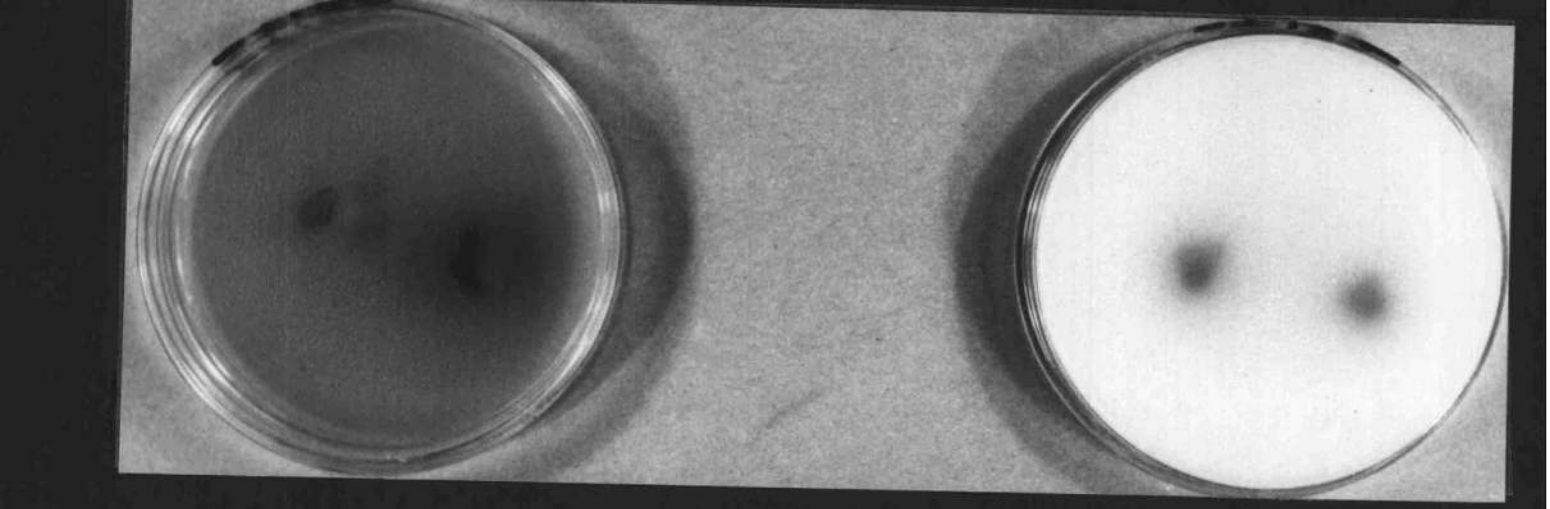
d
d

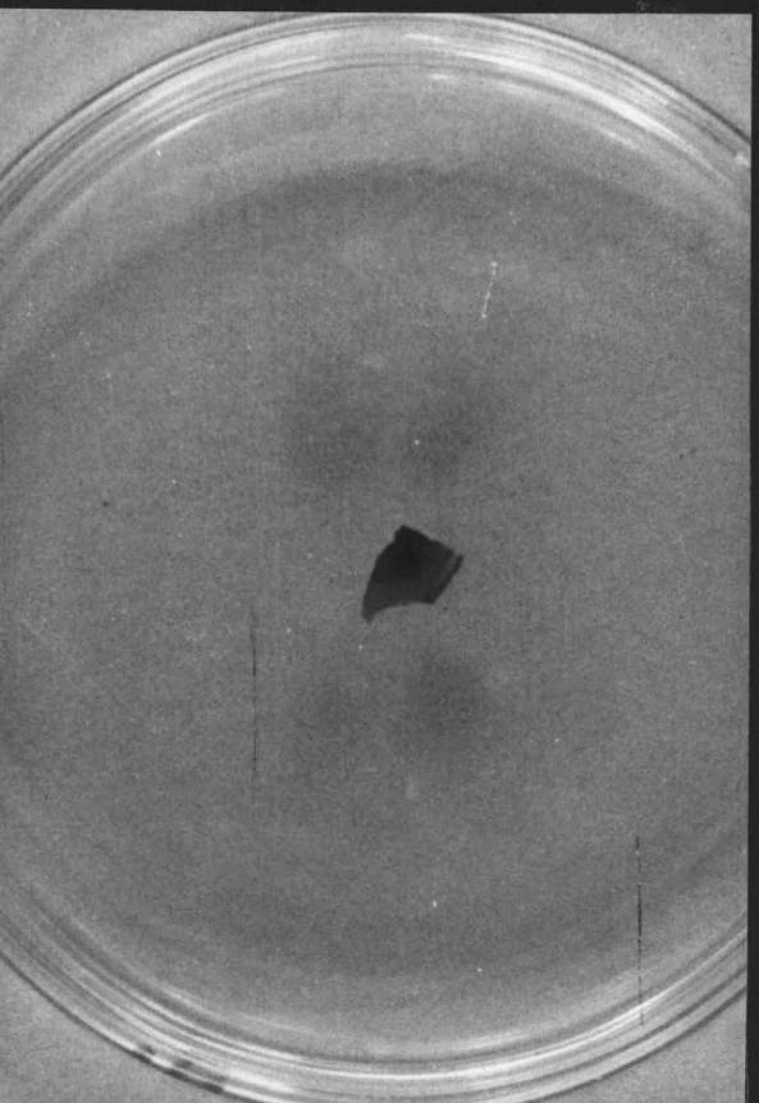
PLATE 47

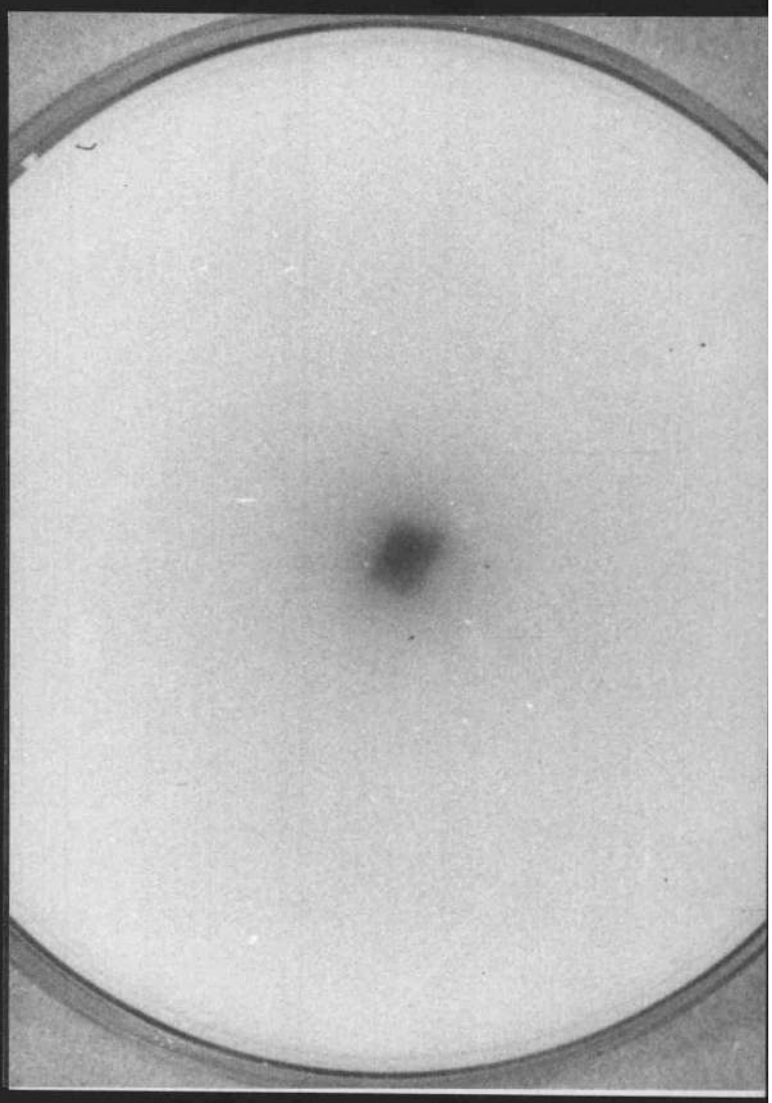
E 1 on tannic acid (right) and gallic acid (left) for 5 days. No growth and only slight positive reaction on both media.

PLATE 48

A 1 producing no growth and only slight positive reaction on tannic acid (right) and gallic acid (left) after 5 days.







All the fungi, except P. betulinus, failed to grow. Davidson et al (1938), pointed out that very intense reaction was usually accompanied by absence of growth.

Discussion

The results described above, indicate that Psathyrella ammophila, C. dunensis, M. grammopodia, E 1 and A 1, can produce polyphenol oxidases to different extents. Following Bavendamm's generalization this would indicate that these fungi are lignin decomposers or white rot fungi while Peziza ammophila is not able to decompose lignin. It was shown in Section 4c(ii) that it is not a cellulose decomposer either, so it would not fall in the category of a brown rot fungus.

Kirk and Kelman (1965) showed experimentally that although certain wood rotting basidiomycetes could not utilize phenols in agar media e.g. Polyporus dichrous, Poria taxicola and Stereum frustulatum, they were able to decrease the lignin content of wood considerably. They surmised that certain compounds which were present in wood or wood degradation products were necessary to induce phenoloxidase production in these species and they concluded that the production of an extracellular phenoloxidase was not a requisite for lignin decomposition.

Lindeberg in 1948 repeated Bavendamm's experiment using 63 species among which were 18 spp. known to form mycorrhiza. He found that only a few mycorrhizal basidiomycetes produced diffusion zones and they did so particularly on gallic acid agar. Boletus subtomentosus, a facultative mycorrhiza former, gave strong oxidative reaction on both media and it was also

able to decompose 49% of the dry matter in sterilized litter in 185 days. Lactarius deliciosus which forms mycorrhiza with Pinus sylvestris and Picea abies also gave pronounced oxidative reaction. From the results of this experiment Lindeberg proved that polyphenol oxidase was produced regularly and abundantly by litter decomposing basidiomycetes which attacked lignin and cellulose, whereas many of the mycorrhizal forming basidiomycetes produced none or just small amounts of the enzyme. The transitional types e.g. Boletus subtomentosus, which forms fruit bodies without the attachment of their mycelia to the host, gave positive reactions. He was not able to draw a distinct boundary between the litter decomposing and the mycorrhizal groups.

4d Decomposition of Native Lignin and Cellulose

Lindeberg in 1944 estimated gravimetrically the amount of decomposition of cellulose and lignin in litter which was caused by soil inhabiting hymenomycetes e.g. Marasmius sp. He used sterilized dead pine needles, leaves of beech and aspen and also the straw of Glyceria maxima in 250ml Erlenmeyer flasks and estimated the amount of decomposition after seven months.

Since some of the sand-dune fungi were found to produce polyphenol oxidases on gallic and tannic acids as shown in the experiment just described, it was decided to investigate their ability to decompose native lignin and cellulose using Lindeberg's (1944) gravimetric method.

Method

Leaves of Elymus arenarius which were dry but still attached to the stem, and the old roots of the same species were collected from Tentsmuir in May, 1967. They were taken to the laboratory where they were washed to remove the adhering sand grains. They were then airdried and cut in small pieces about 1 or 2 cm in length. In 250ml Erlenmeyer flasks were put 5 grams leaves or roots and 50ml distilled water. They were plugged with cotton wool and were autoclaved at 15 lbs. pressure for fifteen mimutes. Glucose at the rate of 10 grams per litre was added to the flasks and this served as starter glucose. The flasks were then inoculated with 4 x 5mm^2 discs from actively growing edges of C. dunensis, M. grammopodia, Psathyrella ammophila, Peziza ammophila and E 1. They were incubated at 23^{o}C.

Results

All five fungi started to grow but growth was extremely slow except for Psathyrella ammophila. After nine months incubation, growth was so slight even in the case of Psathyrella ammophila that when observed visually it was quite obvious that very little or no decomposition had taken place in any of the species and so no gravimetric determinations were made.

Discussion

Melin (1948) found that the hymenomycetes forming mycorrhiza with forest trees were unable to develop on cellulose as the sole carbon source although they were able to produce cellulose splitting enzymes. They were able to grow on sterilized litter only after inhibiting

substances were removed and a nutrient solution containing glucose was added. Perhaps the leaves and roots of Elymus arenarius should be leached before they are used as substrates in case there are any inhibitory substances present. It would be expected that even if the fungi could not use the lignin or the cellulose, they would have grown more vigorously until the supply of glucose was diminished. On the other hand, no other nutrients were added and the fungi failed to develop probably because of their absence. Their inoculum potential (the energy of growth of a fungus available for colonization of a substrate at the surface of the substrate to be colonized, Garrett, 1956) was, therefore, not sufficient for them to attack the lignin. This does not mean that they would be unable to utilize lignin and cellulose in nature since the conditions in the sand-dune are completely different from those in the laboratory.

4. Spore Germination

The purpose of this exercise was to find a medium on which each fungus germinated readily. Therefore, no attempt was made at getting statistical results, the percentage of germination was not calculated and the experimental conditions were not carefully controlled. The criterion used in deciding that germination had occurred was the production of a definite germ tube. In all cases where germination did occur, the germ tubes continued to grow to appreciable lengths but their progress was not further observed except in Psathyrella ammophila where clamp connections were observed in less than two weeks.

In August 1967, fruit bodies of Psathyrella ammophila and Peziza ammophila were collected; those of M. grammopodia and C. dunensis were collected in October of the same year. Spore prints of these were made on clean glass slides as soon as they arrived in the laboratory and except where otherwise stated, the experiments were carried out within eight hours. All incubations were carried out in the dark at the temperatures stated under each experiment. Experiment E was performed in 1966.

Experiment A

Method

Two agar media were made up:-

(1) 2% malt extract agar

(2) Lange's medium (Morten Lange, 1952)

Morten Lange's modification of Kauffman's medium

Difco agar	10-15 grams
Maltose	5.0 "
$MgSO_4 \cdot 7H_2O$	0.5 "
$Ca(NO_3)_2$	0.5 "
K_2HPO_4	0.25 "
Peptone	0.1 "
Distilled water	900 ml
Decoct of horsedung*	100 ml

The horsedung decoct was prepared as follows: 1 fresh "horse-apple" was boiled for 1-2 minutes in 150ml water and 100ml of the filtrate was added to the medium before sterilising at 120°C for fifteen minutes.

*In this experiment 10 grams hare pellets were used instead of Lange's "horse-apple". Hare pellets were used because hares live in the area and their pellets are very common on the dunes. It was thought that although the fungi are not coprophilous their growth might be benefitted by the water soluble constituents of the pellets. It is hardly likely that any substance which is in "horse-apple" and is not present either in hare pellets or in the sand itself, could be influencing germination and growth of the fungi since "horse-apples" are never present in the area. Throughout this experiment the medium is referred to as Lange's modified medium.

Spores from each fungus were allowed to fall from bits of the fruit bodies on to the agar in petri plates from a height of about 10mm. The fruit bodies were removed after an hour or so, depending on the quantity of spores which were released, and they were incubated at 23°C.

Result

There was no germination on medium 1 after a period of four weeks. C. dunensis had an extremely low percentage of germination on Lange's modified medium after eight days. Spores of Psathyrella ammophila started germination after two days on Lange's modified medium and by eight days had almost 100% germination (Plate 49).

Experiment B

Method

The same media which were used in Experiment A were used in Experiment B. Bits of gills of the basidiomycetes and the hymenium of Peziza ammophila were put directly on the agar surface and incubated at 23°C.

Result

Spores of Peziza ammophila germinated on Medium 1 but the percentage was much higher on Lange's modified medium. Some spores which were still enclosed in asci commenced germination after two days (Plates 50-52). This fungus was, therefore, not subjected to further investigations.

Experiment C

Method

Spores of Psathyrella ammophila, M. grammopodia and C. dunensis were put in small vials containing tap water, distilled water, sterilized distilled water and hare dung decoction (10 grams boiled in 150ml distilled water and filtered). The vials were stoppered and incubated at 23°C.

Result

In twenty four hours spores of C. dunensis had germinated profusely in tap water. Germination also

PLATE 49

Spores of Psathyrella ammophila germinating on Lange's modified medium (x100).

PLATE 50

Germinating spores of Peziza ammophila on Lange's modified medium (x60).

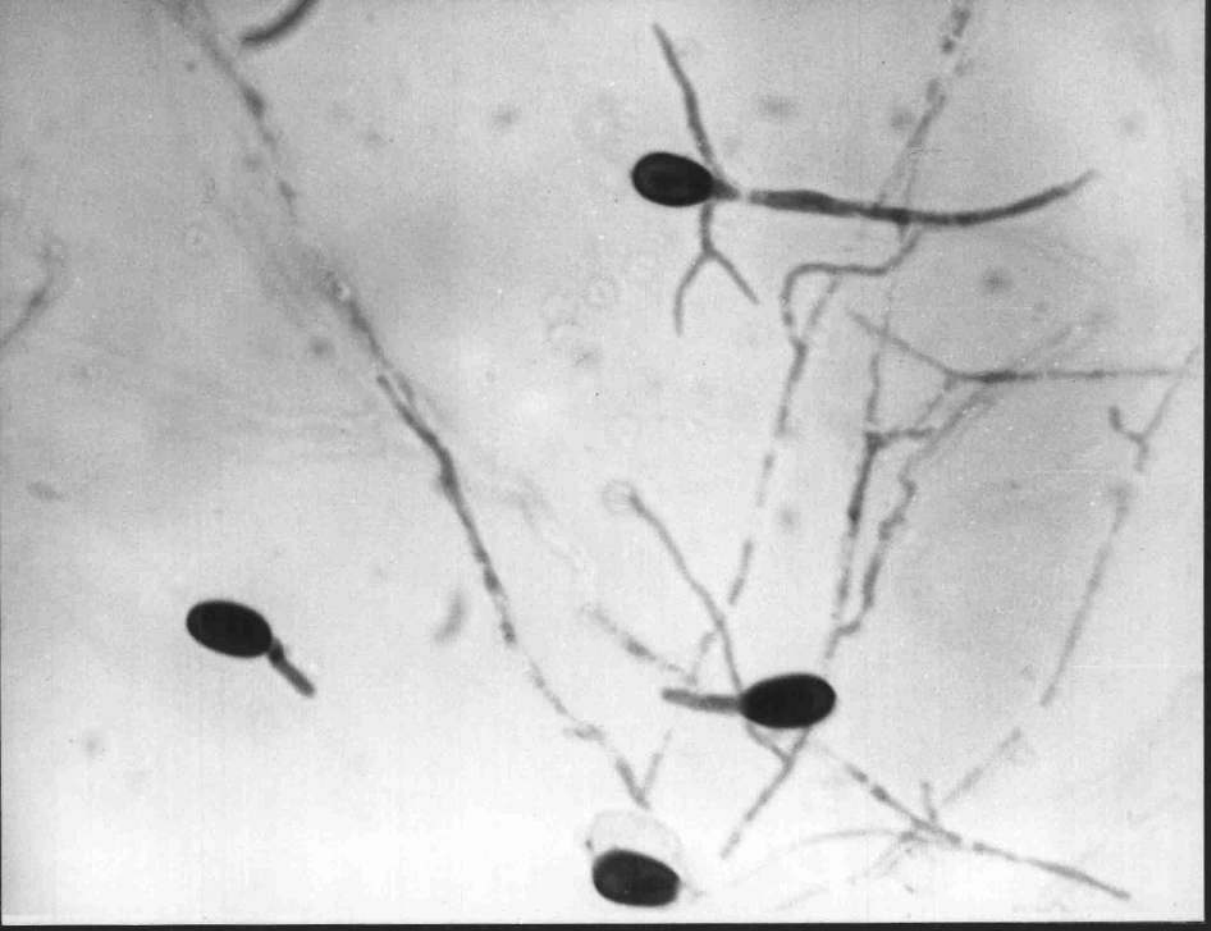


PLATE 51

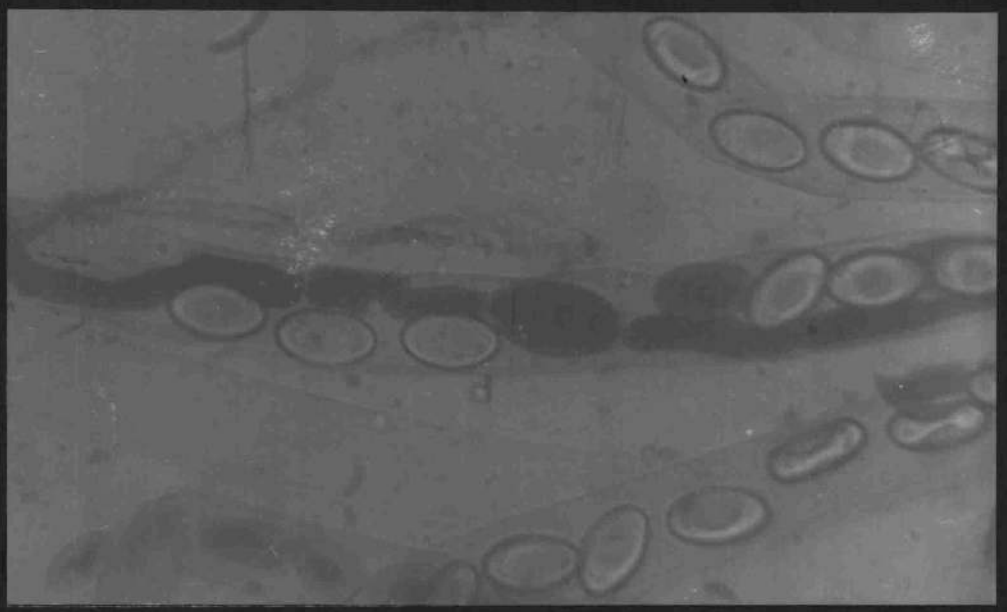
An ascus of Peziza ammophila with eight spores, one of which has started to germinate. (x400).

PLATE 52

A spore of Peziza ammophila with two germ tubes in an ascus (x400).

PLATE 53

Spores of C. dunensis germinating in tap water. (x100).



took place in hare dung decoction but not to the same extent as in tap water. After two days, germination commenced in distilled water and in sterilized distilled water after six days (Plate 53).

The other fungi, i.e. Psathyrella ammophila and M. grammopodia failed to germinate even after six weeks incubation.

Experiment D

Method

Watling's technique, (Watling, 1963a) was then tried on M. grammopodia, the only fungus which has defied all the tests used so far. This involves keeping the spores in a saturated atmosphere at 27°C for twenty four hours, shaking them in sterile distilled water and then distributing them on a nutrient agar consisting of Lange's modified medium. The plates were then incubated at 25°C.

Result

There were no germinating spores after a period of four weeks.

Experiment E (1966)

Method

Spores of Psathyrella ammophila and M. grammopodia which had been in the laboratory for two days were transferred from spore prints which were made on glass slides to petri plates containing the following media:-

3.9% Potato dextrose agar

2.8% Oxoid Nutrient agar (CM3)

1.7% Cornmeal agar

2.0% Washed difco agar

2.0% Unwashed difco agar

Hagem malt agar

Hagem malt agar (Norkrans, 1950) consists of the following constituents:-

Glucose	5.0 grams
$MgSO_4 \cdot 7H_2O$	0.5 "
KH_2PO_4	0.5 "
NH_4Cl	0.5 "
$FeCl_3 \cdot 6H_2O$	5.0 mg
Malt extract	5.0 grams
Agar (difco)	15.0 "
Distilled water	1000 ml

Except for the Hagem malt agar, these media were arbitrarily selected, being available in the Botany Department Chemical Stores.

The spores were removed from the glass slides by means of a sterilized inoculating loop which was also used to disperse the spores over the agar surface. On half the number of plates there were enough spores present to make them readily visible by the naked eye. On the other half the number of spores was so few that their presence could be detected only with the aid of a microscope. This was done so that if the spores produced an inhibitor or a stimulator, its concentration in the agar would be less in the plates with fewer spores and its presence would be detected by the difference in the percentage of germination. Smart (1937) cited the work of Wilson and Cadman who found that spores of Reticularis lycoperdon secreted autocatalytic agent and a relatively high concentration was necessary for successful germination. The plates were incubated at 25°C and 15°C and daily observations were made.

Results

Spores of M. grammopodia failed to germinate on all the media after being incubated for a period of six weeks.

After three days Psathyrella ammophila spores started to germinate only on the nutrient agar at 25°C. Germination commenced on the same agar at 15°C after five days. Apparently, the concentration of the spores did not affect the rate of germination but the lower temperature caused a delay in the germination. Even after six weeks the spores had not germinated on the other media.

Experiments A and C were repeated using spores of M. grammopodia which had been in the laboratory for one year but all failed to germinate.

Discussion

Spores of C. dunensis germinated in tap water in twenty four hours. It took an increasingly longer time to germinate and fewer spores did so on hare dung decoction, distilled water, sterilized distilled water and Lange's modified medium. It did not germinate on malt extract agar when it was incubated at a temperature of 23°C for four weeks. Its poor germination or lack of germination on semi-solid media and better germination on all the liquid media which were used, suggest that it needed more water than was present in the agar media. Perhaps the spores needed liquid water to stimulate the metabolic processes involved in germination and this would not be present in the agar media. An inhibitory factor might have been present in the agar (Fries, 1966) or the spores may themselves produce an inhibitor which

is water soluble and would be more readily removed in water than in the semi-solid media. This suggestion, however, does not account for the better and quicker germination in tap water than in sterilized and unsterilized distilled water but it leads to a further suggestion that the stimulating effect which acts either in stimulating germination or depressing the effect of an inhibitor was partially removed during distillation and sterilization. However, further experimentation is necessary under more controlled conditions.

Psathyrella ammophila was successfully germinated on only two of the twelve media tested i.e. on Lange's modified medium and on Oxoid Nutrient agar. This medium consists of the following constituents:-

"Lab - lemco" Beef extract	1 gram
Yeast extract (Oxoid L 20)	2 "
Peptone (Oxoid L 27)	5 "
NaCl	5 "
Agar	15 "

pH 7.4

It would be interesting to carry out further experiments and find out, if possible, which substance is the stimulator. Since the constituents of the Nutrient agar are known, a series of experiments could be done in which one of the constituents is left out; temperature and pH remaining constant. By checking the percentage of germination on the different media it might be possible to pin-point the activator or activators.

M. grammopodia has so far defied all efforts to germinate its spores. Fries (1966) obtained good results in the germination of Hymenomycetes with a

malt extract agar medium on which living colonies of Rhodotorula or some other unnamed micro-organism had been inoculated. Some species of Hymenomycetes and Gasteromycetes, however, could not be induced to germinate with any of the activators which he tested. He suggested that an inhibitory factor is present in extracts of malt, yeast or other natural products which can be removed by the activity of suitable co-cultivated organism. Schroth and Hildebrandt (1964) suggested that spore germination in the rhizosphere occurs as a result of exudation of carbonaceous and nitrogenous compounds principally amino acids and sugars. The requirements of spore germination of M. grammopodia might be closely associated with its particular habitat and it might be induced to germinate using micro-organisms or living roots as the activator.

The results of these experiments point to the fact that Peziza ammophila germinated well when bits of the hymenium were placed on malt extract agar and Lange's modified medium; that C. dunensis germinated readily in tap water; that Psathyrella ammophila germinated on Oxoid Nutrient agar and Lange's modified medium and that M. grammopodia has failed to germinate under all the experimental conditions used. However, the work here described and the suggestions made and the conclusions drawn, are in no way regarded as final. The scope of the experiment is much too great for hasty conclusions to be drawn but it is hoped that the experimental evidence will encourage further work in this field.

The only certain thing is, although all four fungi grow together on the sand-dune their requirements for spore germination are all different.

SECTION 5

INTERACTIONS OF FUNGI AND GRASSES

5a Production of Synthetic Mycorrhiza

(1) Method

A preliminary test was carried out in 1966 with C. dunensis and the three grasses growing under similar conditions as will be described below. In that test, "seeds" and seedlings in which the plumules had not yet burst through the coleoptiles, were inoculated with the fungus.

The roots were examined after the first, second or third leaves had appeared, but no infection was observed at any of these stages. Kryuger (1961) observed that vesicular-arbuscular mycorrhiza was absent from the roots of grasses at the first leaf stage and was present only as separate hyphae at the third leaf stage. He concluded that the formation of mycorrhiza had seasonal rhythm. This observation led to the necessity of setting up the experiment for a longer period of time and involving all five fungi.

The roots of seedlings of A. arenaria, A. junceiforme and E. arenarius were inoculated with pure cultures of C. dunensis, M. grammopodia, Psathyrella ammophila, Peziza ammophila and E 1, between January and April, 1967. The plants were grown in sterilized sand in earthenware flower pots for eight months after which time the root systems were examined for infection.

Psathyrella ammophila and Peziza ammophila were grown on freshly prepared malt extract agar for 1-1½ weeks prior to use. The others had to be subcultured for three weeks since they were slow growers.

The seedlings used were obtained using the technique described under Method. Those of A. arenaria

and E. arenarius grew vigorously and had long roots with numerous root hairs. They were chosen for inoculation when the aerial parts were between 5 and 7 centimetres in length. Those of A. junceiforme had to be carefully selected as a large proportion of them had roots which were less than 5 mm in length and the plumules failed to burst through the coleoptiles.

The sand and pots were sterilized as described in Section 2b(i)(f). When properly cooled the sand/vermiculite mixture was aseptically transferred to the pots. Sterilized Hoagland's solution (Thomas, Ranson and Richardson, 1956) was added until the sand mixture was thoroughly moistened. Segments of agar with the actively growing mycelium were stirred in the sand and one seedling was transferred to each pot. The roots were arranged so that they were touching the inoculants to ensure contact with each other. A layer of sterilized dry sand was spread over the top and moistened with distilled water (McArdle, 1932).

One of the difficulties of this experiment was the problem of having the seedlings and the fungi at the right stage of growth at the same time that the pots and the sand were ready for use. Agropyron seedlings caused most concern as there was no guarantee that all the caryopses would germinate and when they did germinate that they would be satisfactory for use in the experiment. Peziza ammophila also caused a certain amount of concern when it failed to grow on various occasions. The experiment had to be set up over a period of three months because of the problems just mentioned and also because the pots and sand which were used had to be

sterilized in small quantities at a time and because the sterilization times were so long.

Consequently, this experiment was not repeated although the results are so nebulous that it is difficult to arrive at proper conclusions.

Design of the Experiment

The experiment was set up in two series (Series 1 and Series 2) and for each treatment there were tenfold replications as shown below:-

Series 1

10 pots	with	Ammophila arenaria alone - Control	
10 "	"	Agropyron junceiforme alone - "	
10 "	"	Elymus arenarius alone - "	
10 "	"	A. junceiforme + C. dunensis	This was repeated for all five fungi
10 "	"	A. arenaria + C. dunensis	
10 "	"	E. arenarius + C. dunensis	
10 "	"	C. dunensis alone - Control	
10 "	"	sand alone - Control	

Series 2

The same procedure was adopted as for Series 1 except that the sand was treated in a different manner as described in the Method.

The 480 flower pots were put in green houses and watered on alternate days with distilled water. Once in every two weeks Hoagland's solution was used instead of water. Distilled water was used to make up the Hoagland's solution.

The plants were kept under these constant conditions for a period of eight months after which time they were removed from the pots. The roots were examined for the presence of infection (see Method) and the aerial parts were dried in an oven at 100^{o}C until constant weight was obtained. Their dry weights were then recorded.

5a (ii) Results and Discussions

The results and discussions will be divided into three parts for simplification.

(a) Sporophore Production

Although growth of the grasses appeared to be normal, it was not possible to know whether the fungi were growing or not. Fruit-body production would confirm that they had produced good growth and it would suggest

(i) that the temperature, pH and soil nutrients were appropriate,

(ii) that the roots had not excreted toxic substances which had killed the mycelia and

(iii) that if they were mycorrhiza which depended on their hosts for their nutrients they had made contact with the roots.

However, the absence of fruit bodies would not necessarily mean that the fungi had not grown profusely enough to support fruit bodies, but it would suggest that the requirements for fruiting were unfulfilled probably because one or more of the three conditions mentioned above was unfavourable.

It was thought that in Series 2, the environment in which the fungi were put to grow was nearer to their natural habitat than in Series 1. Steam sterilization would not have changed it as radically as three hours of autoclaving at 15 lbs. pressure. In Series 2 plant remains were present in addition to a living plant and the chief differences between this environment and that in nature were the absence of micro-organisms and probably the addition of simpler nutrients which resulted from hydrolysis of the more complex ones.

If the fungi were soil inhabiting saprophytes then they should grow more vigorously in Series 2 than in Series 1 and consequently produce more fruit bodies. On the other hand, if they were mycorrhizal or obligate parasites, the production of sporophores should be equal in the cases where a plant was present and there should be none in the control pots. Also, if the grasses had any stimulating or inhibiting effect on the fungi, it would be revealed in rate of sporophore production.

Results

It was observed that fruit bodies were produced only in the pots with C. dunensis and Psathyrella ammophila. In Psathyrella ammophila only two sporophores grew. These were in two different pots both with Agropyron junceiforme which were growing in Series 2. The fruit bodies appeared four months after the time of inoculation. Below is a table showing the number of pots in which fruit bodies of C. dunensis grew.

TABLE 6

Sporophore production in Conocybe dunensis

	Control	Agropyron	Ammophila	Elymus
No. of pots with at least 1 in S1	10	7	9	7
No. of pots with at least 1 in S2	10	5	7	6

It is interesting to note that in both series fruit bodies grew in all the control pots which had Conocybe alone. In all the other cases sporophores grew in more pots in Series 1 than in Series 2. In the experimental pots sporophores were produced in 9 of the

10 pots with Ammophila in Series 1 and only 7 in Series 2. In Agropyron and Elymus the results were not different being 7 in Series 1 and 5 and 6 respectively in Series 2. These figures refer to the first crop of fruit bodies which were produced after inoculation. The number of sporophores ranged from 1 to 5 in one pot. In the cases where more than 1 was produced, they appeared in one or two days after one another. Eight months after inoculation, fruit bodies appeared in 9 pots in Series 2: this was first fruiting in 4 of the pots and second fruiting in the other 5 pots. All were in pots which contained a plant.

Since no fruit bodies appeared in the control pots with sand alone, it can be assumed that (i) sterilization was effective in killing all the spores and mycelia of C. dunensis which might have been in the sand and plant debris and (ii) the fruit bodies occurred as a result of the deliberate inoculation of the pure cultures.

Discussion

From the above suggestions, it is very tempting to say that Psathyrella ammophila was a soil saprophyte since it produced sporophores in Series 2 and not in Series 1 and also that Agropyron had some stimulating effect upon fruit body production. It is perhaps more than a coincidence that both fruit bodies were produced under these similar experimental conditions and although the result cannot be regarded as conclusive, it should not be overlooked.

On the sand-dune in Tentsmuir C. dunensis is more common on the fixed dune where Ammophila predominates.

In this region it grows in uncolonized sand and also in close association with the three grasses. It cannot be assumed, however, that any of the grasses had any direct influence on the number of sporophores which were produced since from the experimental results sporophores were more frequent in the absence of the grasses than in their presence. Webley, Eastwood and Gimingham (1952) in their rhizosphere microflora study in the sand-dunes at Tentsmuir, found that Ammophila stimulated the growth of a group of Gram negative non sporing short rods whereas Agropyron did not. Hassouna and Wareing (1965) experimentally showed that the fixation of atmospheric nitrogen was an important factor in the nutrition of Ammophila. They pointed out that under field conditions plants of A. arenaria are heavily infected with mycorrhiza and that it is possible that the mycorrhizal associations played an important part in nitrogen fixation. Nicholson (1959 and 1960) observed the presence of vesicular arbuscular endophytes in A. arenaria and A. juncciforme but his study was not extended to show whether this fungus was involved in the fixation of nitrogen or not. The ability of C. dunensis to fix atmospheric nitrogen has never been tested, but if it cannot, then it is quite possible that it is deriving benefit from the work of the bacteria in the rhizosphere of A. arenaria. This would account for its presence in areas where this grass flourishes. It might also have a preference for this region because of the presence of more decomposing plant materials.

The results also show that more fruit bodies were formed in Series 1, in which the sand was sieved and autoclaved than in Series 2 where the sand was not

sieved, extra bits of dead plant parts had been added and sterilization was done by steaming. Superficially, this points to the fact that the decomposing plant material was not beneficial to the fungus, but this was not the case. In the first few months after inoculation the fungus would have used up all the available nutrients in the pots. This includes those which were originally in the sand and those which were made available by sterilization. Fruit bodies were then produced in both series. At that time the dead plant material would not have undergone much decomposition to be of great benefit to the fungi. However, as the process of decomposition progressed it provided suitable substratum for the growing fungus. Evidence of this was seen in the production of a crop of fruit bodies in Series 2 eight months after inoculation. Therefore, the plant material had a delayed beneficial effect on the growth, survival and reproduction of C. dunensis in the soil and it can be classified as a soil saprophyte (Garrett, 1956). It is assumed here that in Series 1 the fungus did not survive for a long time judging from the absence of fruit bodies in later months.

Romell (1938 and 1939) concluded that certain mycorrhizal fungi would never produce sporophores unless their mycelia were directly attached to the living roots of the hosts. He suggested that the mycorrhizal fungi were wholly dependent on the roots of trees for their energy substrates. With this in mind, it can be concluded that C. dunensis is not an obligate mycorrhizal fungus since it fruited readily in the absence of a

living plant. This tends to agree with Watling's statement (Watling 1963b) that members of the Bolbitiaceae, to which Conocybe belongs, are non-mycorrhizal.

Below is a table which illustrates the fruiting time of Conocybe under the conditions of the experiment. It shows that the time taken for the first fruit bodies to appear in the pots was different.

TABLE 7

Fruiting Times

Averages are shown in brackets

	Control	Agropyron	Ammophila	Elymus
Fruiting time in weeks in S 1	4-9 (5.7)	7-10 (8.29)	6-15 (9.78)	4-9 (5.86)
Fruiting time in weeks in S 2	5-9 (7.6)	8-10 (9.9)	7-13 (9.86)	8-11 (9.5)
		32	32	32

Statistical analyses were done to find if these average times were significantly different. The F-test was applied to find if there were significant differences between the variances. Then applying the appropriate t-test for the significance of differences between the averages, at the 5% level of significance, it was shown that there were significant differences between some of these figures, (Tables 8, 9 and 10).

TABLE 8

Significance of Differences in the Fruiting Times in Series 1

Fruiting times significantly different at the 5% level	Fruiting times NOT significantly different at the 5% level
Control and *Agropyron junceiforme* Control and *Ammophila arenaria* *A. junceiforme* and *A. arenaria* *A. arenaria* and *E. arenarius*	Control and *Elymus arenarius* *A. junceiforme* and *A. arenaria*

These differences suggest that *A. junceiforme* and *A. arenaria* were exerting some influence on the time of fruiting but *E. arenarius* was not.

TABLE 9

Significance of Differences in the Fruiting Times in Series 2

Fruiting times significantly different at the 5% level	Fruiting times NOT significantly different at 5% level
Control and *E. arenarius* Control and *A. arenaria* Control and *A. junceiforme*	*A. junceiforme* and *A. arenaria* *A. junceiforme* and *E. arenarius* *A. arenaria* and *E. arenarius*

Therefore, all three grasses were exerting some influence on the fruiting times in this series.

TABLE 10

A Comparison of the Significance of Differences in the Two Series

Fruiting times significantly different at the 5% level	Fruiting times NOT significantly different at the 5% level
Control S 1 and Control S 2 *A. junceiforme* S 1 and S 2 *E. arenarius* S 1 and *E. arenarius* S 2	*A. arenaria* S 1 and *A. arenaria* S 2

Therefore, the influence of *E. arenarius* and *A. junceiforme* on *C. dunensis* in Series 1 was different from that in Series 2.

In the case of *M. grammopodia*, *Peziza ammophila* and *E 1*, no fruit bodies were formed in any of the pots in the experiment. The conditions with which the fungi were provided were obviously not suitable for sporophore production; perhaps not for vegetative growth either. This could be as a result of one or more of a number of factors, for example, soil pH and soil moisture (Kouyeas, 1964; Taylor and Parkinson, 1964) and soil temperature (Roa, 1959; Rouatt, Peterson and Katznelson, 1963). The grasses might have been exuding substances which were deleterious to the fungi or under the experimental conditions they were unable to exude any stimulatory substances which would be beneficial to the fungi (How, 1941; Osvald, 1948; Melin and Rama Das, 1954; Rovira, 1956 a, b, c; Rovira and Harris, 1961 and Dobbs and Gash, 1965). Sterilized soils have been found to be toxic to plants (Gerdemann, 1964).

Perhaps in nature the fungi rely on other micro-organisms for some essential substance - a vitamin or growth factor, and since the micro-organisms were removed by sterilization, these substances would be absent (Melin and Rama Das, 1954; Lochhead, 1957). It is also possible that an unfavourable quantitative balance of constituents would also lead to the absence of sporophores.

5a (ii) (b) Dry Weights of the Aerial Parts

In this section an attempt was made to correlate the dry weight of the aerial parts of the grasses with the effect of the fungi on their growth. Four methods have previously been used to determine the effect on the plant of the fungus-root association. They were:-

(1) Field observations. This is usually inaccurate because no allowance can be made for all the other factors which are simultaneously influencing the development of the plant.

(2) Anatomical structure of the infected roots.

(3) Micro-chemical analyses of infected and non-infected plants.

(4) Experimental method whereby plant roots are brought into contact with the mycelium of a fungus and, by measuring the seedling height, leaf length and observing the foliage colour etc., direct evidence is obtained on the effect of the fungus on the plants. (McArdle, 1932).

Investigators who used (3) agree that mycorrhizal fungi are parasitic and found no evidence of symbiosis. Masui (McArdle, 1932) found that the fungi removed from the roots all the amino acids, most of the carbohydrates, tannins and nitrates, some phosphorus, potassium and ammonium. Young fruiting bodies contained large amounts of these substances. The evidence from many workers who used (4) was almost opposite to (3). McArdle (1932) found no absolute proof that the presence of mycorrhiza was either detrimental or beneficial to conifer seedlings. Bain (1937) attempted to find out whether

mycorrhiza derived nutrients from cranberry roots and arrived at the conclusion that the fungus tended to reduce or intensify the toxicity of the medium which was harmful to the host plants. Richards and Voigt (1964) reported that inoculation of Pinus radiata with Rhizopogon roseolus increased the nitrogen of seedlings even where it did not increase the dry weight. Gerdemann (1964) found that plants of maize which were inoculated with arbuscular-vesicular mycorrhiza grew more vigorously and appeared more healthy than uninoculated ones.

In the present investigation dry weight of the aerial parts of the grasses was used in determining the effect of the fungi on the plants. On the whole there were no obvious differences between the size and colour of the plants. The root systems were not included in the dry weight because they were examined for fungal infections (see part C). The aerial parts were separated from the root systems at the hypocotyl region and were dried to constant weight at 100°C.

Some of the grasses with Peziza ammophila died after they had been growing for four months. The greatest loss was with Ammophila arenaria where only two plants survived. It is the writer's opinion that the fungus did not cause the death of these plants. They were in a separate greenhouse from the other pots and during the summer months it gets very hot reaching a temperature of 90-95° Fah. The sand became very hot and watering could have resulted in steaming the roots, particularly because they were in these small pots. Frequent repetition of this would undoubtedly kill the plants.

In this phase of the experiment the control pots had no fungal inoculations except for those which might have survived the process of sterilization or those which were later introduced by air currents. The effect of these fungi would have balanced out since they would have also been present in the inoculated pots.

Results

Tables 11 and 12 show the average dry weights in grams of all the three grasses grown in pots which were inoculated with the five fungi in both Series 1 and 2.

TABLE 11

Dry Weights of Aerial Parts of Grasses in Series 1 in grams

(The number of plants which survived are shown in brackets)

Fungi / Grasses	Control	Conocybe	Melanoleuca	Psathyrella	Peziza	E 1
A. juncei-forme	1.51 (8)	2.29 (8)	3.30 (9)	2.51 (10)	1.78 (2)	3.95 (8)
A. arenaria	0.52 (9)	1.43 (10)	1.65 (10)	1.88 (5)	0.19 (2)	1.63 (10)
E. arenarius	2.23 (8)	3.03 (10)	3.34 (5)	2.37 (9)	2.08 (6)	2.26 (10)

The averages were again statistically analysed. Because the figures were so few, the comparisons which could be made statistically were limited. However, from the t-test for the significance of differences between the averages at the 5% level of significance, the following have been deduced:-

Series 1

(1) The average dry weight of all the grasses grown with Melanoleuca was significantly higher than the average of all the grasses in the control.

(2) The average dry weight of all the grasses grown with E 1 was significantly greater than the average of all the grasses in the control.

(3) The average dry weight of all the grasses grown with (a) Melanoleuca and (b) E 1 were significantly higher than the average of all those grown with Peziza.

In all the other possible combinations, there was no significant differences between the averages in the overall dry weights.

The results, therefore, show a statistical probability that Melanoleuca and E 1 had some stimulatory effect over the control on the dry weights of the grasses as a whole. These averages, however, were not significantly different from those with the other fungi, except Peziza, and so the effect can be regarded as negligible. Unfavourable environmental conditions may be responsible for the significant difference observed with Peziza.

TABLE 12

Dry Weights of Aerial Parts of Grasses in Series 2 in grams

(The number of plants which survived are shown in brackets)

Fungi / Grasses	Con-trol	Cono-cybe	Melano-leuca	Psathy-rella	Peziza	E 1
A. juncei-forme	1.92 (4)	2.47 (8)	2.46 (9)	1.28 (4)	1.46 (6)	1.52 (6)
A. aren-aria	1.06 (2)	1.08 (10)	1.29 (5)	0.90 (4)	1.02 (2)	1.87 (5)
E. aren-arius	1.49 (3)	3.09 (8)	2.23 (9)	1.63 (4)	2.60 (8)	1.88 (7)

In this series, the average dry weight of all the grasses grown with C. dunensis was significantly greater than the average of all the grasses grown with Psathyrella ammophila. In all the other combinations there were no significant differences.

Taken as a whole, there was a significant difference between the average of all the grasses in Series 1 and those in Series 2, those in Series 1 giving higher averages.

Discussion

A microscopic study of the anatomy of the roots of the three grasses shows that the endodermis consists of 2-3 layers of highly lignified cells. This acts as a barrier to fungal infection of the conducting tissues. During the study of infection, no hyphae were observed in the endodermal cells nor in the stele, nor in the actively growing root region. Nicolson (1959) reported the absence of vesicular-arbuscular

endophytes in these tissues as well. The parts of the roots which are responsible for the absorption of water and mineral salts, and the conduction of these to the aerial parts are not infected so that these functions are not obstructed by the presence of hyphae in the roots. Therefore, the presence of the fungi in the cortical cells should not have any deleterious effect on the dry weight of the aerial parts.

Although hyphae were seen in cortical cells, there was no sign of digestion to suggest exchange of material or a case of parasitism. Also, if the fungi contributed to the nutrition of the grasses, it would be necessary for the fungi to have good supply of external mycelia to take up the material from the soil. This was at no time observed.

Zak (1964) pointed out that ectotrophic mycorrhizal fungi may protect the roots in four ways - (1) by utilizing the surplus carbohydrates and thus reducing the attractiveness of the root to pathogens, (2) by serving as a physical barrier to infection, (3) by secreting antibiotics and (4) by favouring along with the roots, protective rhizosphere organisms. Extending these functions of mycorrhizal fungi to the fungi of the sand-dune is perhaps not advisable, but a fungus like E 1 could very well be performing at least the first two of the four functions.

Harley (1957) cited the work of Brook (1952) and Morrison (1957) where they both showed that Pernettya seedlings were stimulated in growth by mycorrhizal fungi especially on nutrient-poor soils.

Harley and McCready (1952), Harley and Brierley (1954 and 1955) demonstrated the uptake of phosphate by mycorrhizal roots and the distribution of this substance between host and fungus. The sand-dunes are very poor in nutrients especially nitrates, phosphates and potassium (Willis and Yemm, 1961; Willis 1963 and 1965; Oliver 1967). Even if the fungi under investigation are not contributing to the nutrient supply of the grasses, the experimental results do not suggest that the host-fungus relationship is detrimental to the host.

5a (ii) (c) Root Infection

The production of sporophores by Conocybe and Psathyrella in the pots, is visual proof that the mycelia were actively growing, but it does not indicate whether the roots of the grasses in those pots and in the pots with Melanoleuca, Peziza and E 1 were infected or not. Therefore, microscopic examination of the individual roots was carried out as direct evidence of the degree of infection which took place.

The method used for the determination of infection was described in Section 2(b)(ii) and some of the observations are shown in Tables 13 and 14 and Plates 57-64.

Results

General Observations

(1) A general pattern of growth was observed with the roots of all the grasses. They grew to the bottom of the pots producing very few laterals. From the bottom they turned outwards and grew very closely around the side of the pots so that the greater part of the root system was confined to the lower third of the pots.

(2) Majority of the plants of A. junceiforme had roots which were brown in colour and appeared to be very unhealthy under all the conditions of the experiment. In the other two grasses brown coloration was present in patches along the length of the roots. This discoloration was not associated with the presence of fungi.

(3) The cortices of many roots and especially the brown ones were very easily separated from the central stele.

(4) Except for those grasses which were grown with E 1, all the infections seen in the roots were confined to

When internal, they were chiefly intracellular and did not cause any obvious damage to the cells, nor to the roots as a whole.

In the roots of Agropyron, infection was heaviest in those which were associated with the fruit bodies. Even then it was confined to the first 4cm of the crown region of the older roots.

Roots Inoculated with Peziza

In culture this fungus has no distinguishing features and it was impossible to tell whether the hyphae were those of Peziza or those of a contaminant.

Roots Inoculated with E 1

In Ammophila roots and roots of Agropyron and Elymus where the infection was not severe, hyphae were present in the outer cortex. Some cells were filled with hyphae but the cell walls were still present. Some roots had no hyphae in the two outermost layers of cells.

Very heavily infected areas were seen macroscopically as white regions ("lesions") on the primary roots of Elymus and Agropyron (Plates 54-56). A transverse section of one of these lesions shows the hyphae occupying the whole of the cortex except for the two outermost layers of cells. The majority of cell walls had disappeared in the region occupied by fungal hyphae, probably as a result of enzymes secreted by the fungus after the hyphae had entered the cells. Some lesions completely encircled the stele while others formed incomplete circles. It would appear, that the fungus spreads laterally as well as longitudinally from the point or points of infection.

PLATE 54

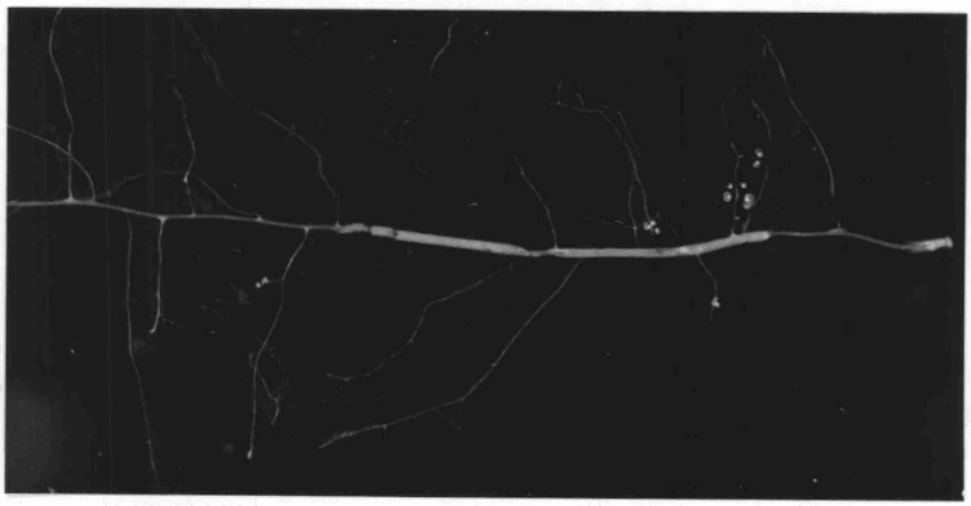
Roots of A. junceiforme showing patchy decortication (a) and white lesions (b) caused by E 1, (x 1).

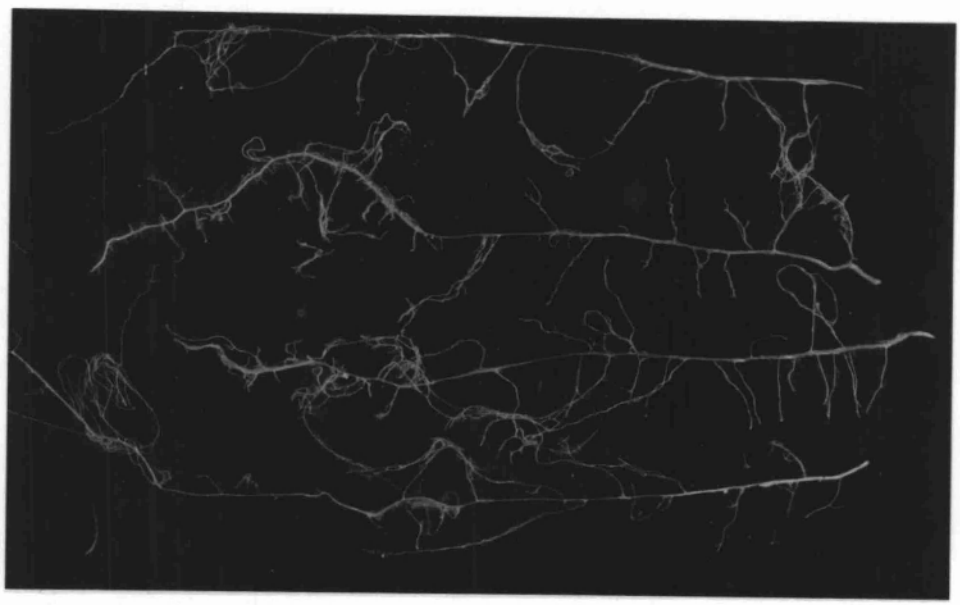
PLATE 55

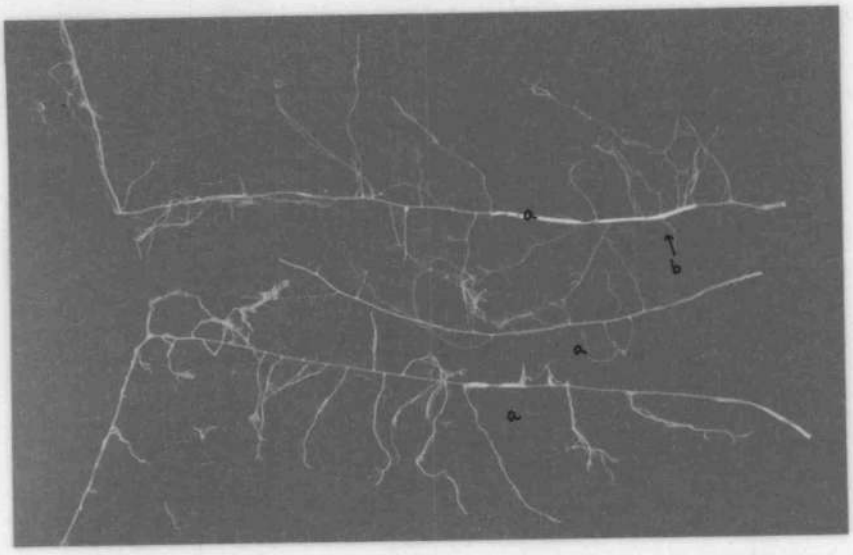
A root of A. junceiforme illustrating decortication after infection by E 1(c), (x2).

PLATE 56

Roots of E. arenarius showing decortication (a) and white lesions (b), (x 1) caused by E 1.






a
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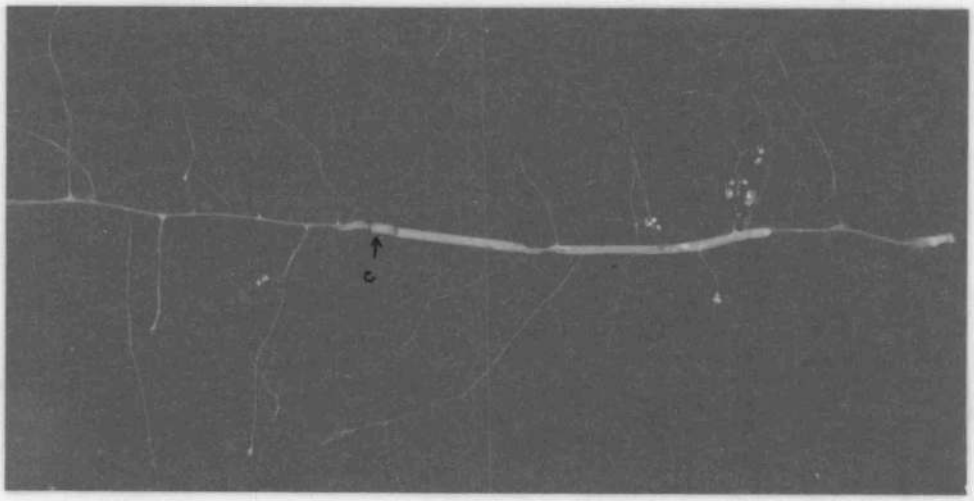
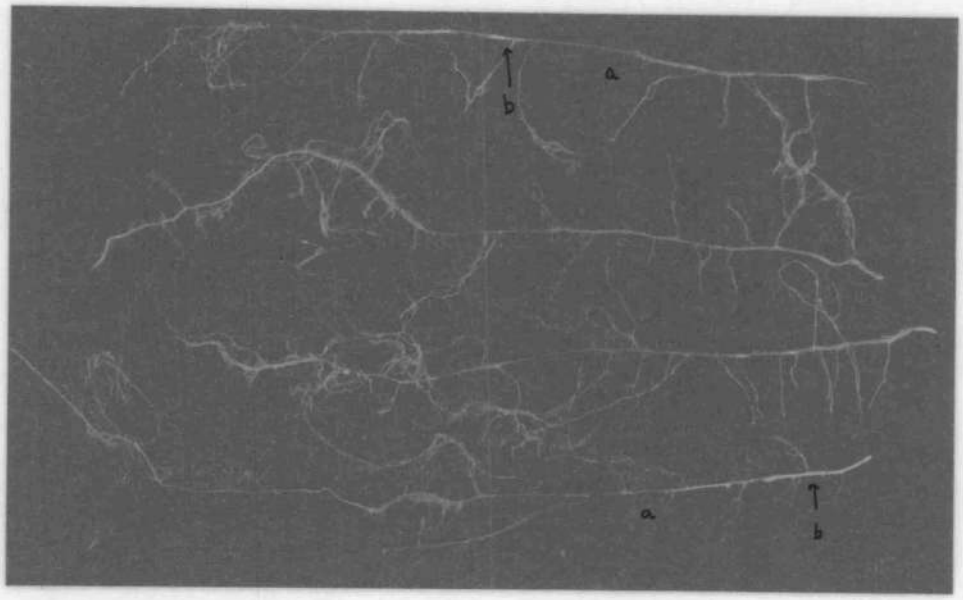
a
a


c


b
a
a
b

In cases where no lesions were present, bulbous hyphae as well as narrow ones were present. From the crown to the tip there was an increase of straight hyphae and a decrease of bulbous ones. One root which was 17cm long had lesions along its entire length. Another one which was 60cm long had a lesion which extended 15cm from the crown of the root. Hyphae decreased in quantity but were present to just behind the tip. Lesions are, therefore, the extreme state of infection which starts at the crown region and slowly works its way down. If given enough time the fungus could probably occupy all the roots.

It was difficult to tell how the fungi passed from one cell to another. No bore-holes were observed, and since the hyphae naturally form constrictions along their length, it was not possible to conclude that they passed through pits by constrictions.

Hyphae were seen in some lateral roots but they appeared to have passed out from the main root.

There was evidence of patchy decortication along the length of the root (Plate 56) and it would appear that after the cortical cell walls have been broken down by the fungus, the entire cortex with the hyphae shreds off leaving the endodermis exposed. Waid (1962) however, observed that the fungi of the outer cortex of decomposing rye-grass roots moved inwards as the roots decayed and he suggested that this was because of the availability of oxygen in the different parts of the cortex.

E 1 behaved like an obligate parasite in that the hyphae were entirely intracellular (Brown, 1936) but differed in that it is easily cultured in synthetic media.

TABLE 13

Root Infection

	Conocybe						Psathyrella						Melanoleuca						Peziza*						Control					
	Agro-pyron		Ammo-phila		Elymus		Agro-pyron		Ammo-phila		Elymus		Agro-pyron		Ammo-phila		Elymus		Agro-pyron		Ammo-phila		Elymus		Agro-pyron		Ammo-phila		Elymus	
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
Roots showing little or no decortication	++	++	+	+	+	+	++	+	+	+	+	++	++	++	+		+	++	+	++	+	+	++	++	+	+	+	+	+	+
Roots showing decortication for more than 5cm	+	+	+	+	+	++	+	+	+	+	++	+	+		++	++	++	+	++	+	++	++			++	++	++	++	++	++
Roots with hyphae among root hairs only		+		+		+	+	+	+	++	+	++					+	+				++	+	+						
Roots with hyphae on and/or in cortex	+	+	+	+	+	+	+	++	+	+	+	+	+		+		++		+	++			+							
Roots showing contamination by other fungi	+	+	+	+	+	+	+				+	+	+	+		+		+							+	+	+	+	+	+

\+ = present in less than 50% of the roots examined
++ = " " more " 50% " " " "

*Hyphae recorded as infection might be contamination

TABLE 14

Infection of Roots of E 1

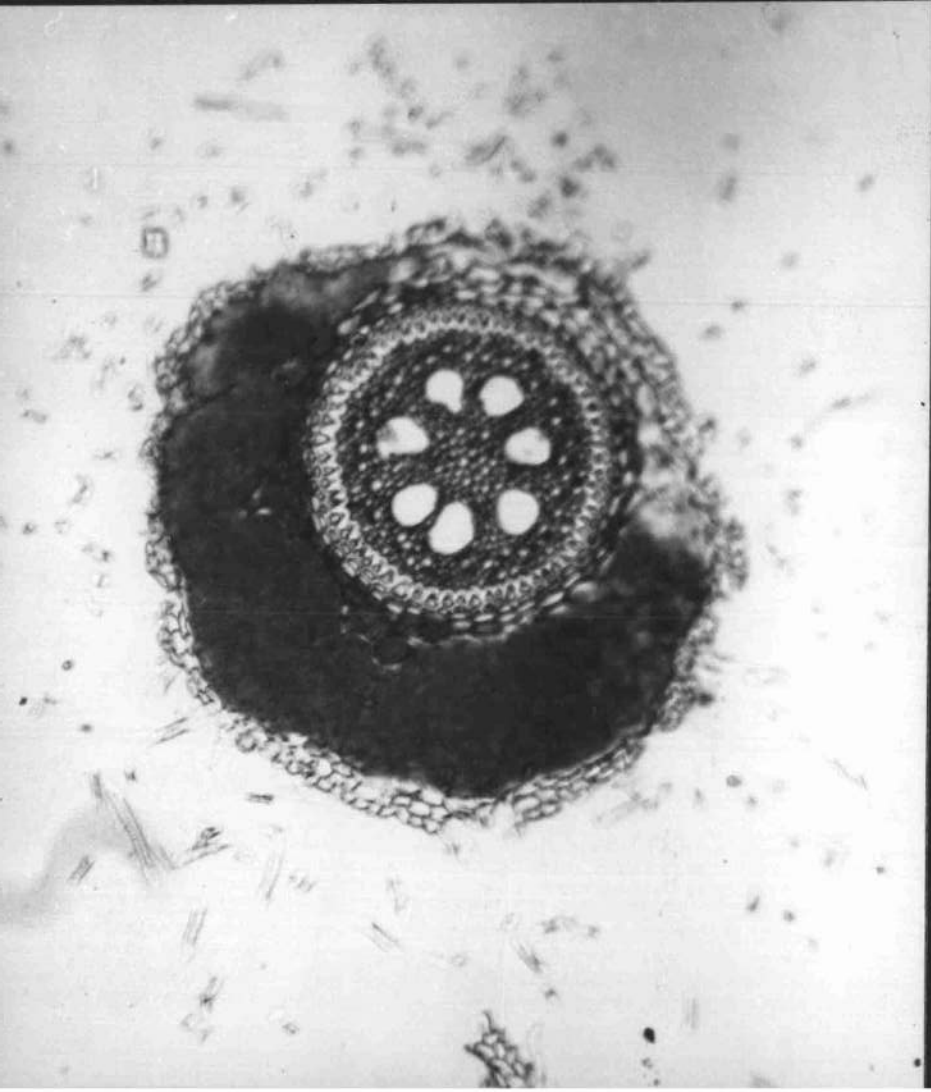
	Agropyron		Ammophila		Elymus	
	S1	S2	S1	S2	S1	S2
% roots showing decortication	50	60	60	20	40	50
% roots showing infection	70	100	20	60	80	100
% roots with white "Lesions"	25	60	-	-	20	40
% roots showing contamination	10	10	10	20	20	20

PLATE 57

T.S. of root of *E. arenarius* showing early stage of infection by *E 1* (x 100).

PLATE 58

T.S. of root of *E. arenarius* at a later stage of infection (x 60).



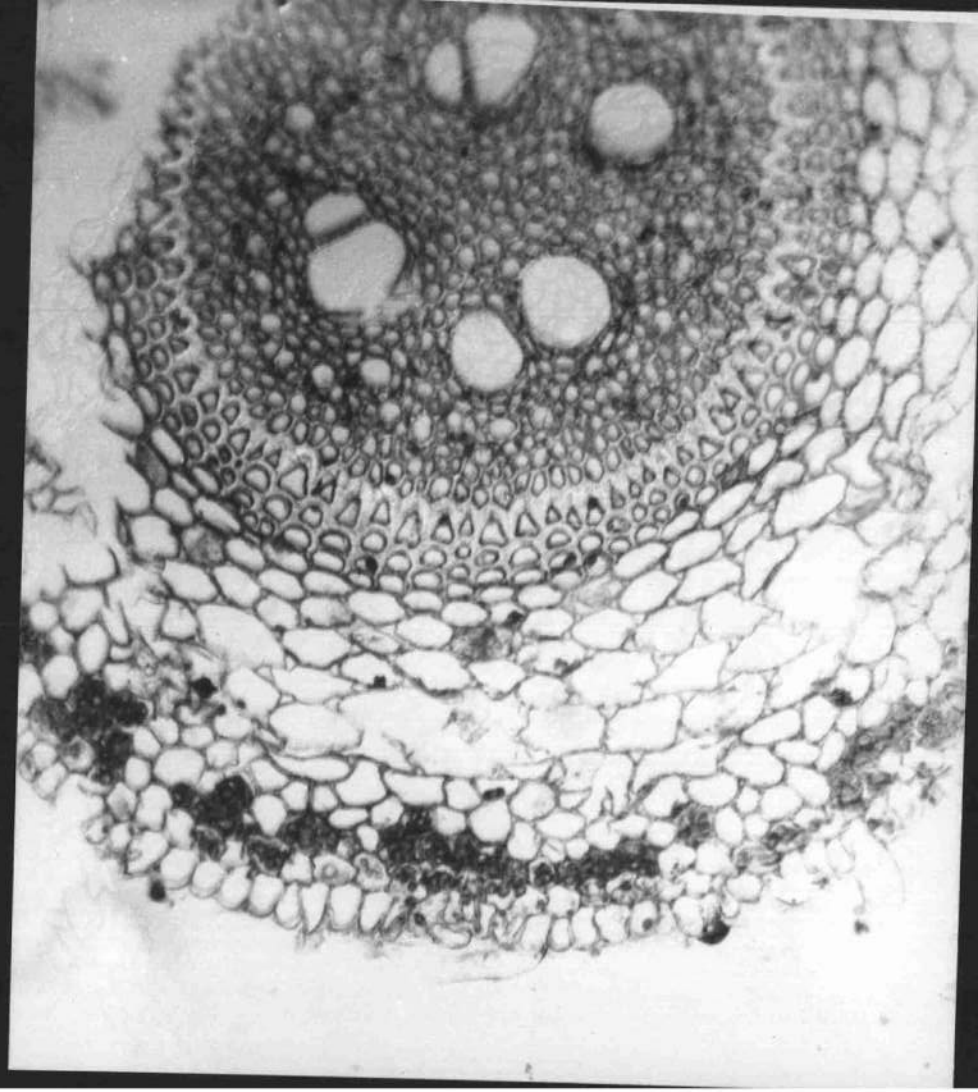


PLATE 59

T.S. across a lesion in A. junceiforme showing :-

(a) epidermis and outer cortex free from infection

(b) cortical cells being invaded

(c) remains of host's cell walls

(d) 3-layered endodermis free from infection (x 400).

PLATE 60

TAngential longitudinal section of root of A. arenaria infected by M. grammodia.

(a) swollen hyphae (x100).

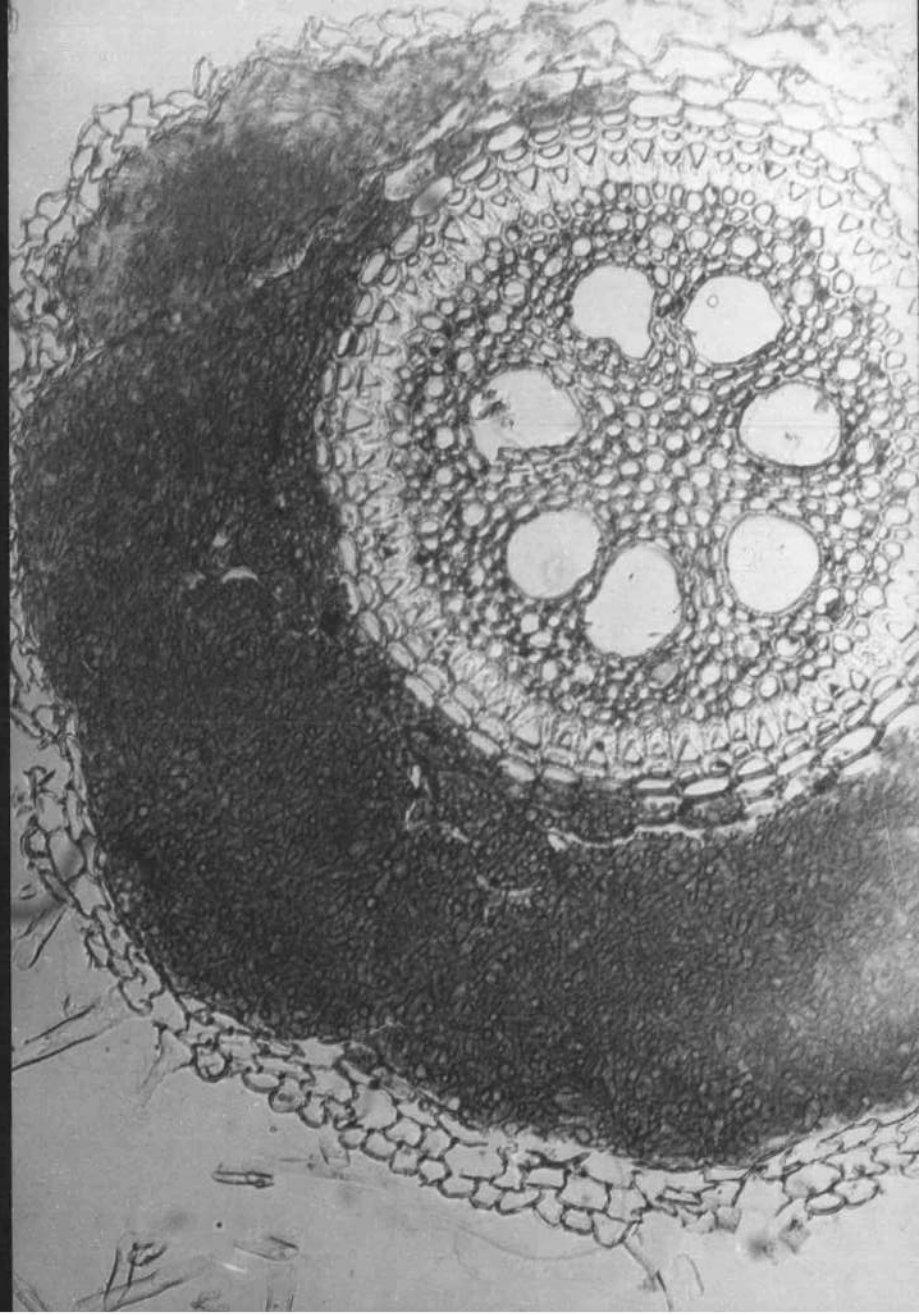
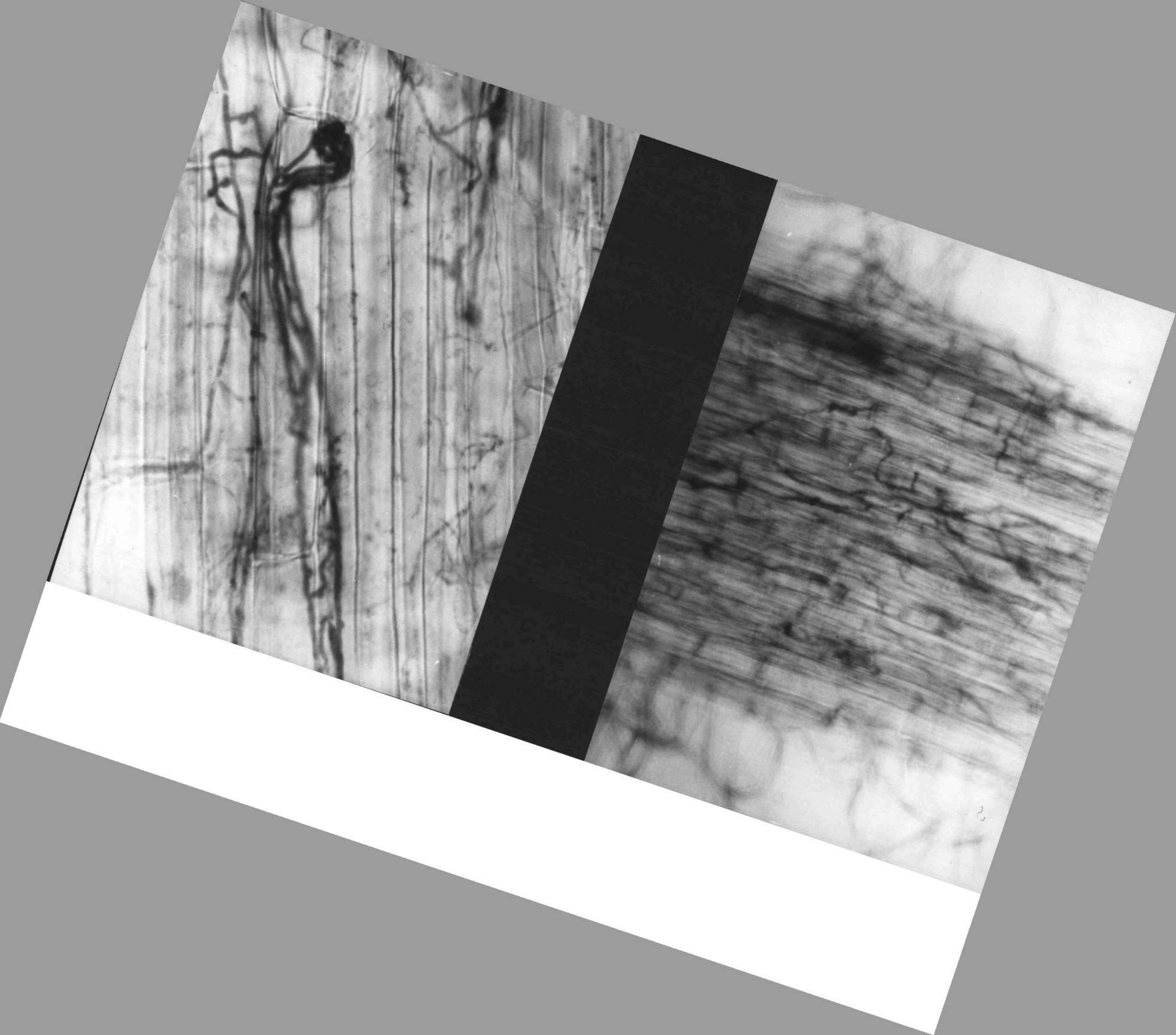


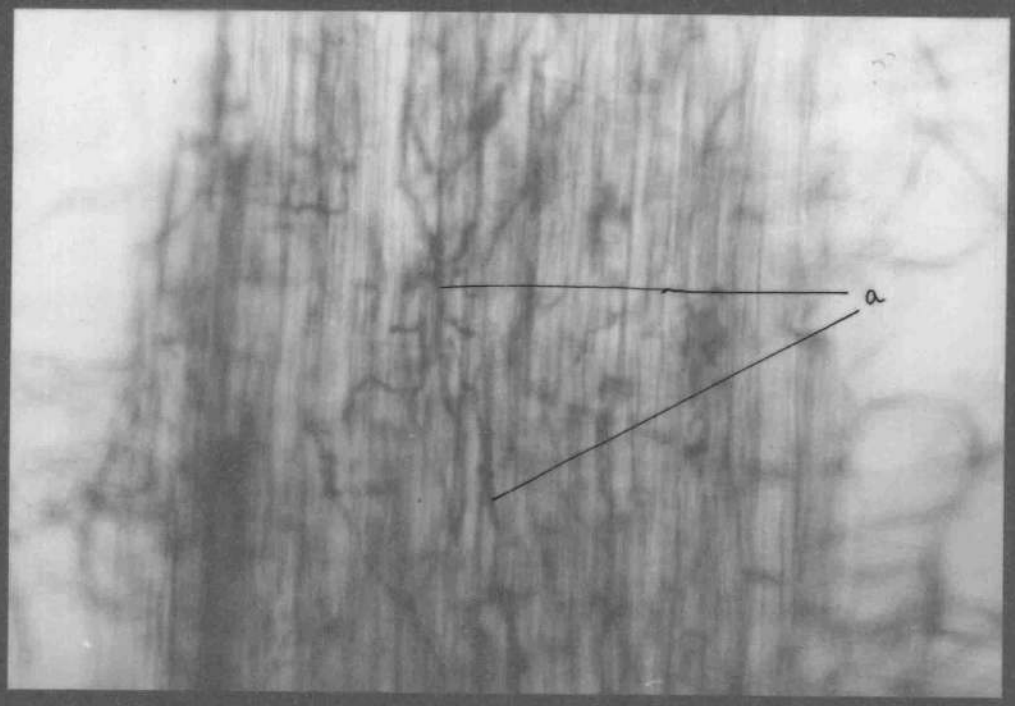
PLATE 61

T.L.S. root of *A. junceiforme* with hyphae of *Psathyrella ammophila* (a), x 100.

PLATE 62

T.L.S. root of *E. arenarius* showing hyphae of *Psathyrella ammophila* in cortical cells (x 400).




a

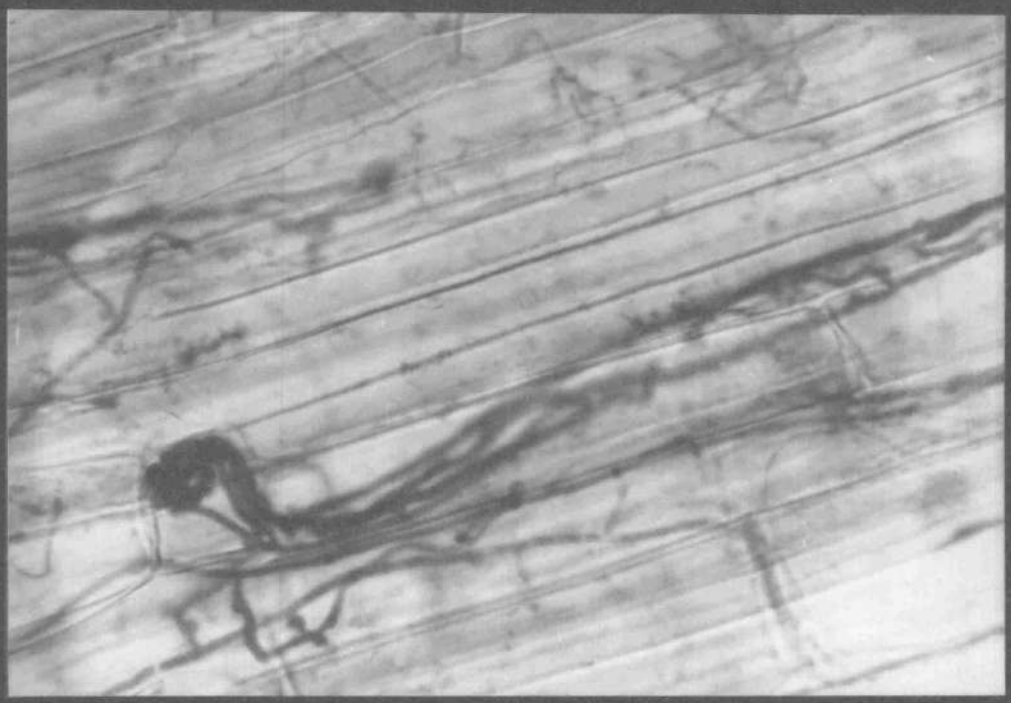
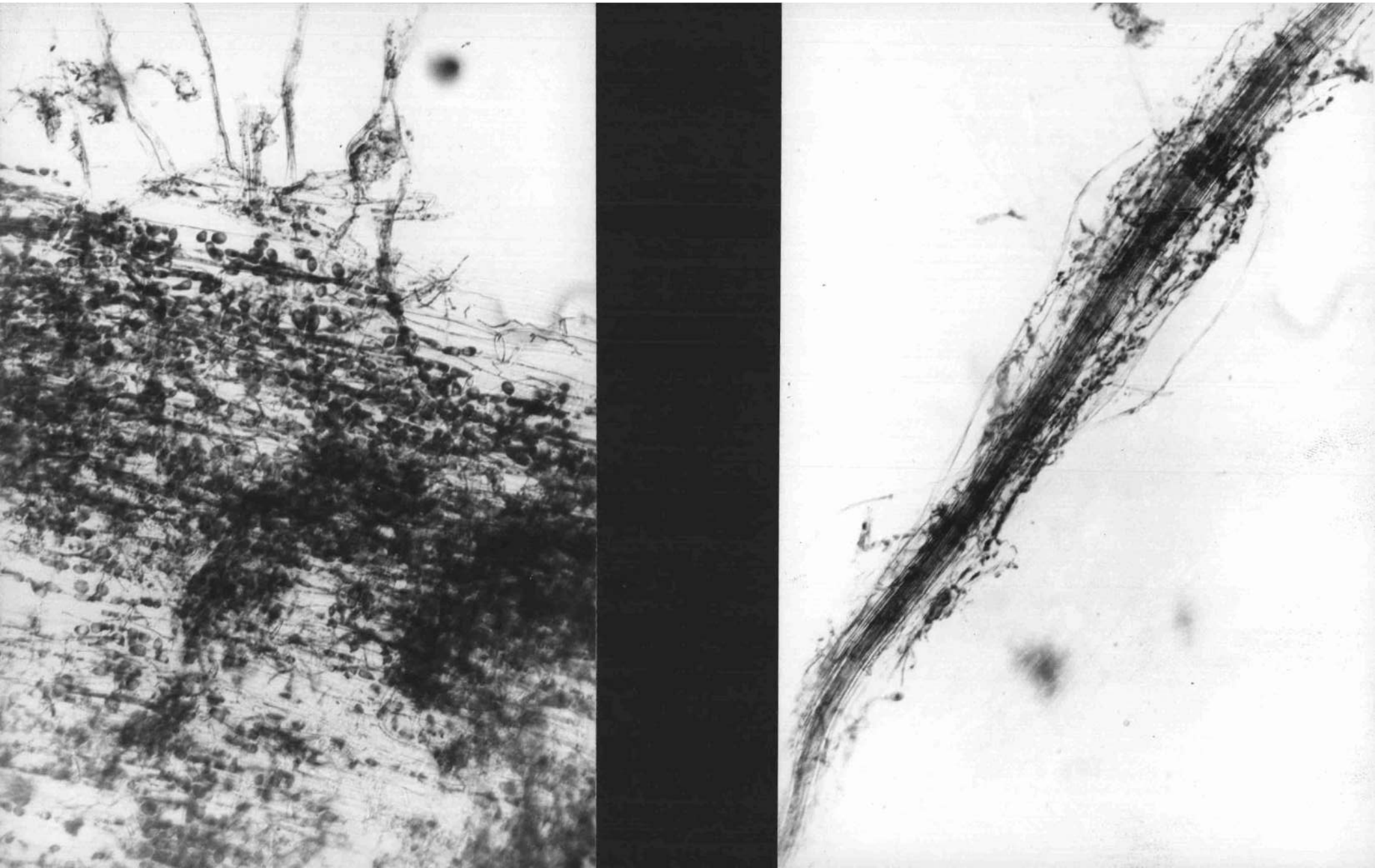


PLATE 63

T.L.S. root of *A. arenaria* heavily infected by *C. dunensis*, x 100.

PLATE 64

Lateral root of *A. arenaria* with hyphae of *C. dunensis* along its length, x 100.



Discussion

It has been realized by many workers that sterilized soils are always somewhat abnormal for the growth of the host and the fungus since they are changed biologically, structurally and chemically and are sometimes toxic (Gerdemann, 1964). Therefore, negative results or abnormal infections do not demonstrate that a fungus is unable to form mycorrhiza (Harley, 1957). The data obtained from pure culture studies and artificial laboratory experiments are not always applicable to the activities of saprophytes or parasites in their natural environment where they are subjected to the diversities of a rhizosphere community. Soil reaction is important and influences root infection according to the moisture content, pH, carbon dioxide and oxygen concentration, toxins and nutrients. Taylor and Parkinson (1964) studied the effect of certain environmental conditions on the development of root surface mycofloras on dwarf bean roots and concluded that soil environment may affect the incidence of fungi on plant roots in three ways; (i) it may have a direct effect on fungal growth in the soil adjacent to the root, (ii) it may affect the growth and metabolism of the root and (iii) it may have a joint effect on the fungus-root relationship.

Kouyeas (1964) reported that fungi grew better in soils with low water content and suggested that the suppression of growth under conditions of high water content may be due to restricted aeration or a high carbon dioxide concentration. Zak (1964) ascribed dying tree rootlets to poor aeration when the soils

were wet. Garrett (1937) did not think that a carbon dioxide concentration which was high enough to retard the growth of fungi would be of common occurrence in soil atmosphere. This, however, does not rule out the possibility of low oxygen and/or high carbon dioxide concentration in a pot of the size which was used in the current investigation.

All tree mycorrhizal fungi investigated with respect to pH have been found to be acidophilic (Melin, 1953) but different species require different pH. Lactarius deliciosus has an optimum pH of 5.5–6.0 (Harley, 1957) while Boletus elegans has an optimum of 4.0–5.5 (How, 1941). Since sterilization can change the pH of soils, then it is likely that the sand mixture in the pots had a different pH from that in the sand-dunes where the fungi thrive. Warcup (1951) found that partial sterilization by steam or formalin appeared to exert a deleterious effect causing profound changes in the microflora and fauna of the soil even after three months. He associated the changes with the fact that sterilization affects the physical properties of the soil and increased the solubility of many nutrients. Sterilization by autoclaving can change the environment by removing the inhibitory effect caused by soil mycostasis on hyphal growth in soil. However, Dobbs and Gash (1965) found that all the investigated sands which were deficient in organic matter i.e. those from mobile dunes and sea strand, exhibited a strong residual inhibition after autoclaving. Both the calcium carbonate and the ferric ions may contribute to the residual mycostasis in these soils.

The conditions of the present experiment are:- the soil was taken from a mobile dune, (therefore, low nutrient supply and possible residual mycostasis), it was autoclaved or steamed (therefore, wrong pH, biological, chemical and physical changes) and the plants were grown under the conditions restricted by the size of a 5" pot, (therefore, low oxygen and high carbon dioxide possibilities). All these adversities to healthy plant growth might have caused poor growth of the fungi and consequently, low infection of the roots.

It has been established that micro-organisms flourish in rhizospheres chiefly because of substances being exuded from the roots. Pearson and Parkinson (1961) determined the sites of excretion of ninhydrin positive substances to be correlated with the centres of high proteolytic activity within the root. Working with broad beans, they found that exudation reached its peak one day after planting and then fell off quickly. However, there was a limited region just behind the tip which continued to exude small quantities of ninhydrin positive substances. The roots of the grasses being studied were confined to the lower third of the pots and the fungi were slow growing. It is very doubtful that they had grown down to the level where their growth could be influenced by the possible exudates. In fact there were no hyphae in association with the young regions of the roots. Furthermore, watering would have caused downward leaching of these substances making them less available to the hyphae. If root exudation is the main factor which determines the growth of fungi in the rhizosphere, then these fungi did not thrive in the pots

because of the absence of root exudates in their environment and their inability to keep pace with the rapidly growing roots.

In Garrett's account of the biology of root infecting fungi, (Garrett, 1956) he states that "infection is a struggle between the offensive forces of the parasite and the defensive forces of the host. The issue of the struggle is decided by the extent of the reserves on either side". At the time of inoculation, both the host and the fungi were actively growing - the hosts being seedlings, were at a very susceptible stage to parasitism. If the fungi were potential parasites and were potentially capable of penetrating the root surfaces, they would have done so at that time if their inoculum potential was enough. A slight delay would enable the fast growing root to escape infection. There was little evidence of them penetrating the roots, and when they did they might have done so actively or passively (the latter will be discussed in another paragraph). If the penetration was active then the offensive forces of the host might have accounted for the low infection. By the time the roots became senescent and less able to ward off infection (Waid, 1957), the fungi had probably lost the capacity to invade the host. Primitive parasites have been defined by Garrett (1956) as those which are so restricted by host resistance that they can infect only seedling host plants, immature or senescent root tissues of older plants, or root systems of plants growing under adverse conditions. The time of infection of the grasses by E l, Conocybe or Psathyrella is not known but the possibility of them being parasites, primitive or otherwise, cannot be

ruled out until further consideration has been given to their growth in the soil.

Decortication is a common phenomenon in roots. As the root ages the root hairs and outer layers of cortex are sloughed off and unless there is the production of cork or lignified tissue this will continue until the entire cortex is lost. This is the situation in the grasses. Gadgil (1965) did not observe vesicles of vesicular-arbuscular endotrophic mycorrhiza in grasses until the grasses were sixteen weeks old. There was strong development of mycorrhizal fungi from 16-40 weeks after germination of the seeds. Decortication did not begin until after thirty weeks. Judging from the degree of decortication observed in the sand-dune grasses, it would have started much earlier in these grasses than in those studied by Gadgil. However, his observation strengthens but does not confirm the suggestion made on page *93* that decortication occurred as a result of infection by E 1. There was no evidence that infection by Conocybe of cortical cells took place before or after decortication so that the above suggestion cannot be extended to cover all the cases of decortication which were observed. Also, there is no evidence to show how or when it was initiated.

During the present study of infections, hyphae were observed among root hairs more frequently than on or in the cortical cells. Failure of these hyphae to infect the roots may be due to lack of growth because of soil conditions which were described in an earlier paragraph. On the whole, they might be indifferent to the presence of the roots, and were on or in the

cortex or among the root hairs because they happened to be in the same environment. This situation was referred to earlier as passive infection. They might also be typical rhizosphere fungi and would be more frequently present in this ecological niche than inside the root. Many workers have studied the distribution of fungi in the rhizospheres of different plants (Harley and Waid, 1955; Chesters and Parkinson, 1959; Parkinson and Clarke, 1961; Taylor and Parkinson, 1961 and 1965; Pugh, 1962 and Parkinson, Taylor and Pearson, 1963).

It is the writer's conviction, however, that the primary reason for absence of infection was a low inoculum potential. Garrett (1956) introduced this term and defined it as "the energy of growth of a fungus (or parasite) available for the colonization of a substrate (or host) at the surface of the substrate to be colonized". The inoculum potential can be increased in two ways:-

(a) by increasing the number of infecting units of the fungus per unit area of the root. In setting up the experiment 12 sq mm of agar with actively growing mycelium was inoculated into each pot.

(b) by increasing the nutritional status of such units. De Bary (1887) was the first to notice that the penetration of the host plant by Sclerotinia sclerotiorum depended on the nutritional status of the fungal inoculum and failed to occur if this was inadequate. Since that time, the subject has attracted much attention.

McArdle (1932) stirred mycelia from cultures made on agar containing only maltose into the sand mixture and

obtained successful mycorrhiza on conifer seedlings. However, How (1942) used chopped glucose agar cultures in her experiment and the fungi failed to establish themselves. She attributed this failure to the agar medium since this becomes exhausted of nutrients before the slow growing mycelia had had time to establish themselves in the soil. There was no mycorrhizal formation when sawdust compost cultures were used either, so she suggested that some soil conditions were necessary for the establishment of mycorrhiza with larch seedlings. Agar inocula were used in the present experiment but it no doubt has the same limitations as those expressed by How (1942).

Garrett (1956) mentioned that infection was unsuccessful when agar was used. He grew the fungus on a suitable medium like a mixture of sterilized soil or sand with maize meal and mixed it in high proportion with the soil. These heavily inoculated soils resulted in successful inoculations.

Therefore, the inoculum potential is probably a function of the number and growth vigour of active hyphal tips per unit of the inoculum and per unit area of the substrate to be infected. If hyphal tips are present and there is not sufficient nutrient supply to give them the energy source necessary for penetration, infection will not take place. Schroth and Hildebrand (1964) related root exudates with infection by suggesting that they provided the pathogen with a temporary supply of nutrients enabling it to vegetate briefly and increase the number of infecting units per unit area of host surface. At the same time they suggested the

possibility of root exudates lowering the inoculum potential by containing toxic substances which inhibit the growth of the pathogen.

Björkman (1949) and Handley and Sanders (1962) have shown that a high ratio of soluble carbohydrates in the roots to the amount of nitrogen and phosphorus available to the plant encourages the formation of mycorrhizal associations. This might also account for the failure to produce infection in culture.

Where the infecting units are organized into mycelial strands and rhizomorphs, Garrett (1956) inferred that this increased the infective power as compared with a single hypha. It was observed that Psathyrella ammophila formed small mycelial strands which meandered among the root hairs. This might have been an attempt at increasing its infective power but some other factor prevented it from doing so on a greater scale. Only if the concentration of effective inoculum per unit area of root surface is sufficiently high to overcome the host's resistance to invasion will a successful and progressive infection occur. Hyphae of Conocybe, Melanoleuca and Psathyrella were seen in cells of the cortex which shows that they were potentially capable of penetrating the walls. It might be argued that they penetrated dead or senescent cells but there is no substantiating evidence for this.

Examination of the roots from the pot experiment, gave the result that infection, except in E 1 was confined to the crown region of all roots. This agrees with the findings of most workers e.g. Chesters and Parkinson (1959) suggested that the frequent occurrence

of fungi in the crown regions resulted from the increased number of dead root cells superimposed on a decreased exudation of organic material from this zone. A similar conclusion was arrived at by Rovira (1965) that moribund root cells and root hairs make significant contributions to the proliferation of organisms in the older portions of roots. If the fungal hyphae penetrated the crown region while the roots were still young and susceptible to infection, the reasons for discontinued growth in the host cells are not clear. There was no sign of digestion to suggest host resistance to their presence. If the fungi colonized the crown region after that region had become senescent, then they must have survived in the soil as soil saprophytes in which case the dead cells of the old root would be new substratum for them to colonize. The question as to whether they are parasites which were living in conditions unfavourable to their growth or whether they are saprophytes has not yet been answered.

The isolate E 1 gave very interesting results. It infected almost all the roots with which it came into contact. It occupied a well defined area of the roots just outside the endodermis and the infection spread for considerable distances along the length of the roots. Environmental conditions were apparently favourable for this fungus. It is probably a parasitic fungus since it was isolated from roots and it was able to produce such strong infections under the same experimental conditions as the other fungi in which infection was very limited.

5b Incidence of Infection in the Field

Roots of A. juncciforme, A. arenaria and E. arenarius were collected in September, 1966 and May, 1967 and were examined for the presence of Basidiomycetes as described in the section on Method.

In September, the active growing season for the grasses is near its end. Nevertheless, there were many young roots on the underground stems. Neither these nor the young parts of old roots were infected by Basidiomycetes. No attention was given to the presence of other fungi which might have been present. The criterion which was used in the identification of a Basidiomycete was the presence of clamp connections. This ruled out the possibility of detecting hyphae of Melanoleuca grammopodia - a Basidiomycete without clamp connections, and any others which fall in that category.

Although the roots in which hyphae bearing clamp-connections were seen were not in a state of obvious decomposition, they had isolated brown patches which, however, were not associated with fungi. The fact that the roots had numerous root hairs which frequently rendered examination of tangential longitudinal sections difficult, suggests that they were still functioning as absorbing organs.

More than 50% of the mature roots which were examined had hyphae with clamp-connections in the cortex. They were especially abundant in the first 4cm from the crown region and decreased in quantity towards the middle of the root and completely absent from the root tip region. At this time of the year all the hyphae were narrow and

no bulbous ones were observed. The endodermis and stele were free from fungal hyphae.

The above description holds true for all three grasses which were examined although all attempts to isolate the infecting Basidiomycete(s) from the roots of Ammophila and Agropyron failed.

In May 1967, the situation was slightly different. This is near the beginning of the growing season and all metabolic processes would be going on at a more rapid rate than in September.

Table 15 shows the number of plants from which roots were taken for examination, and the percentage of infection in them.

TABLE 15

Infection of Roots in May, 1967.

Species of Grass	No. of plants from which roots were examined	% infection by Basidiomycetes
Agropyron junceiforme	12	-
Ammophila arenaria	20	60
Elymus arenarius	15	-

It is interesting to note that at this time basidiomycetous hyphae were present in the roots of Ammophila only although roots of all three grasses were heavily infected by other fungi which had no clamp-connections. It would be unwise to conclude that basidiomycetes were completely absent from roots of Elymus and Agropyron since only 15 and 12 plants respectively were examined.

There were two basidiomycetes infecting Ammophila roots, one with bulbous hyphae and the other with straight hyphae (Plate 14). Taken as a whole, 16% of the root sections produced only basidiomycetous hyphae on agar plates, 20% produced basidiomycete contaminated by bacteria or some unidentified fungi, and 40% of them produced no growth at all.

It has been reported by previous investigators that there is seasonal rhythm in the occurrence of fungi in roots. Kryuger (1961) made observations on the infection of four grasses by vesicular-arbuscular endophytes and found that in spring the degree of infection was less than in summer and it again declined in autumn. He also found that in sandy soil the mycorrhiza developed during flowering in the second year of the life of perennial grasses. Seasonal rhythm could account for the absence of basidiomycete(s) in Agropyron and Elymus roots in May. They probably colonized roots of Ammophila much earlier than the others.

Absence of hyphae in the endodermis and vascular tissue may be due to the highly lignified walls of the endodermis which acts as a physical barrier to infection. Low oxygen concentration in the lower levels of the dunes could account for the presence of basidiomycetes only near the crown regions of the roots. The roots are extensive and penetrate to great depths. Fungi which are sensitive to low oxygen concentrations might, therefore, chose substrates which are in the upper levels of the soil.

Although only three Basidiomycetes were isolated, one from the roots of Elymus and two from Ammophila roots, this does not mean that these were the only three which infected the roots. Failure to obtain isolations from Agropyron and Ammophila roots in September suggests that their nutritional requirements are more specific. They are probably specialized root inhabiting fungi which will not grow readily on synthetic agar plates.

Serial washing of roots with sterilized distilled water has been used to study (i) the active mycelia on living roots and other surfaces in the soil (Harley and Waid, 1955), (ii) the distribution of fungi within the decomposing tissue of rye-grass roots (Waid, 1957) and (iii) the colonization of roots of Pisum sativum by fungi (Stenton, 1958). These studies produced only few sterile Basidiomycetes among the isolates. However, the occurrence of Basidiomycetes in roots of grasses is not an unusual phenomenon. Warcup (1959) isolated an abundant and varied population from wheat roots and from the soil in the wheat field. He was able to induce these to fruit in culture. Apinis (1965) isolated a species of thermophilous Coprinus from debris of coastal grasses including A. juncoiforme and A. arenaria. He also induced this fungus to fruit in culture. Their presence in plant debris and the relative ease with which they grew and fruited on synthetic media suggest that they were not parasites with specific nutritional requirements, but mere isolation of a fungus from dead plant material is not enough proof that the fungus is a soil inhabiting saprophyte, because the plant material might have been invaded while they were still alive.

Therefore, until further evidence is produced, it cannot be concluded that the fungi which were isolated from the roots of A. arenaria and E. arenarius living in Tentsmuir are soil saprophytes.

5c The Effect of Root Extract Agar on the Growth of the Isolates.

The purpose of this experiment was to determine whether or not the roots contained any heat-resistant water soluble substance(s) which might affect the growth of the fungi. An extract was made from the roots, solidified with agar and inoculated with the fungi.

Method

Roots of A. arenaria, A. junceiforme and E. arenarius were collected in Tentsmuir on August 22, 1967. The sand was removed by washing and the roots were airdried and cut in pieces which were about 10mm long. Ten grams of each species were homogenised in a blendor with 100ml distilled water for five minutes and filtered through a bit of gauze. The residue was again blended with 50ml distilled water for two minutes and filtered. Both filtrates were mixed and passed through Whatman filter paper No. 542 in a buchnor funnel.

Within twelve hours the filtrates were incorporated in a basal medium (see the section on vitamins) in the following proportions:-

(a) 1000ml basal medium + 18 grams difco agar = control
(b) 900ml " " + 100ml Agropyron extract + 18 grams agar
(c) 900ml basal medium + 100ml Ammophila extract + 18 grams agar
(d) 900ml basal medium + 100ml Elymus extract + 18 grams agar

The media were autoclaved at 15 lbs. pressure for fifteen minutes and were poured in previously sterilized petri plates.

The fungi which were used in this experiment were C. dunensis, Melanoleuca grammopodia, Psathyrella ammophila, Peziza ammophila, A 1, E 1, Polyporus betulinus and Panaeolus semi-ovatus. They had been growing on 2% malt extract agar for 2-3 weeks. The experiment was set up in triplicate and incubated at 23°C for four weeks.

Methods of Assessing Growth

Growth was recorded in two ways:-

(a) By measuring the diameters (Table 16a)

(b) By obtaining the dry weights (Table 16b)

The methods used in assessing growth were similar to those which Madelin (1956) used in his studies on the nutrition of Coprinus lagopus. He found that "treatment is liable to loss of weight by the escape of soluble materials through cell membranes made permeable by killing, but this loss and, therefore, the weight obtained was assumed to be equivalent to the true weight of the mycelium. The magnitude in loss caused by this treatment was investigated by comparing the dry weights of 23 desiccator-dried, 13 days old mycelia from liquid cultures before and after heating in tubes of water in a steamer at 100°C for thirty minutes. The loss after treatment was 21.3 ± 0.88% of the initial dry weight". This loss in weight applies to the result of this experiment since the same methods were used.

Results

Table 16 (a) and (b) shows the average diameters and dry weights of the fungal colonies after four weeks of incubation. The dry weights were calculated as a

TABLE 16

Growth of Fungi on Root Extract Agar

(a) Diameters of Colonies in mm. Average of 3 replicates. Grown for 4 weeks.

Source of Root Extract \ Fungi	Conocybe	Melano-leuca	Psathyrella	Peziza	E 1	A 1	Panaeolus	Poly-porus
Control	28	22	44	24	48	70	55	73
Agropyron extract	28	30	70	60	80	80	57	80
Ammophila "	28	28	55	40	80	80	60	90
Elymus "	26	30	58	30	80	80	53	80

(b) Dry weights in mg. Incubated for 4 weeks. Dried at 50°C. Average of 3 replicates.

Source of Root Extract \ Fungi	Conocybe	Melano-leuca	Psathyrella	Peziza	E 1	A 1	Panaeolus	Poly-porus
Control	60	8.5	3.4	2.1	18.3	35.9	11.7	27.2
Agropyron extract	94.2	14.9	24.1	34.2	73.1	89.7	52.9	40.5
Ammophila "	78.4	25.7	12.9	4.7	65.9	106.1	71.3	25.0
Elymus "	90.7	21.7	22.4	0.8	44.8	78.4	64.7	49.6

percentage of the controls and the resultant figures are expressed in the form of histograms shown in Plates 65-72.

The average diameters show that C. dunensis grew equally well on all the media including the control. M. grammopodia showed better growth on the extracts than on the control with most stimulation from Agropyron and Elymus. Psathyrella and Peziza were also stimulated to grow better on all three extracts but particularly so on Agropyron. E 1 and A 1 which on malt extract agar are very slow growers, grew very quickly on all media including the control. Panaeolus showed only slight stimulation. Polyporus was also very slightly stimulated on Elymus and Agropyron.

From these results it appears that all the roots caused some stimulation: Elymus being the least effective.

However, the results obtained from the dry weights are quite different. C. dunensis shows a marked stimulation when grown on all the root extracts particularly on the extracts from Agropyron and Elymus. Melanoleuca showed a threefold stimulation when grown on extract from Ammophila, $2\frac{1}{2}$ fold on Elymus and $1\frac{3}{4}$ fold on Agropyron. Psathyrella was stimulated by all three root extracts. Its weight on Agropyron was 7 times, and that on Elymus $6\frac{1}{2}$ times the weight of the control. In Peziza, all the dry weights were very low except for that on Agropyron extract. Bearing in mind that this fungus is rather erratic in its growth pattern, (see the section on Morphology and cultural characteristics), it

PLATE 65

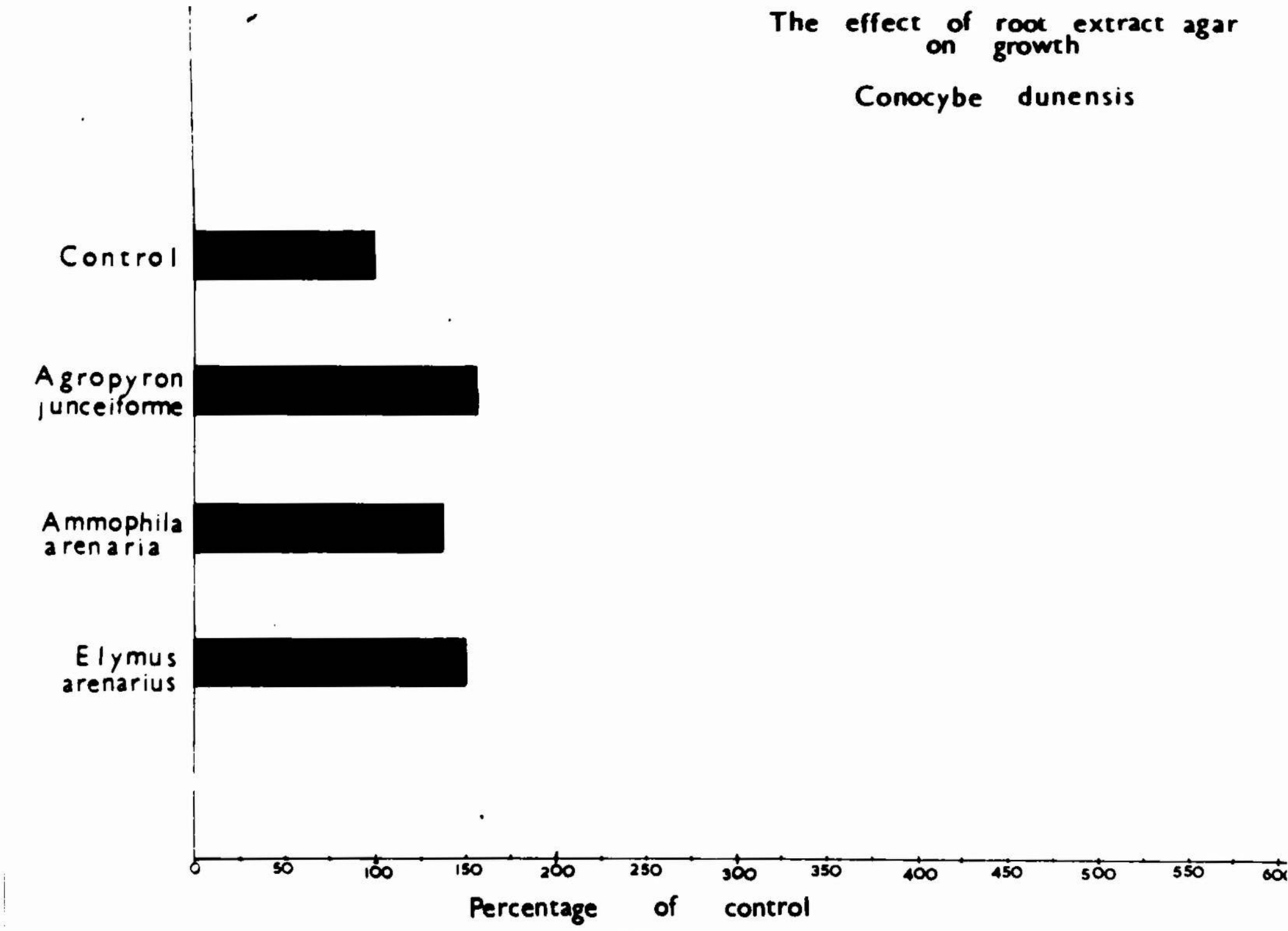

The effect of root extract agar on growth
Conocybe dunensis
Control
Agropyron junceiforme
Ammophila arenaria
Elymus arenarius
0
50
100
150
200
250
300
350
400
450
500
550
600
Percentage of control

PLATE 66

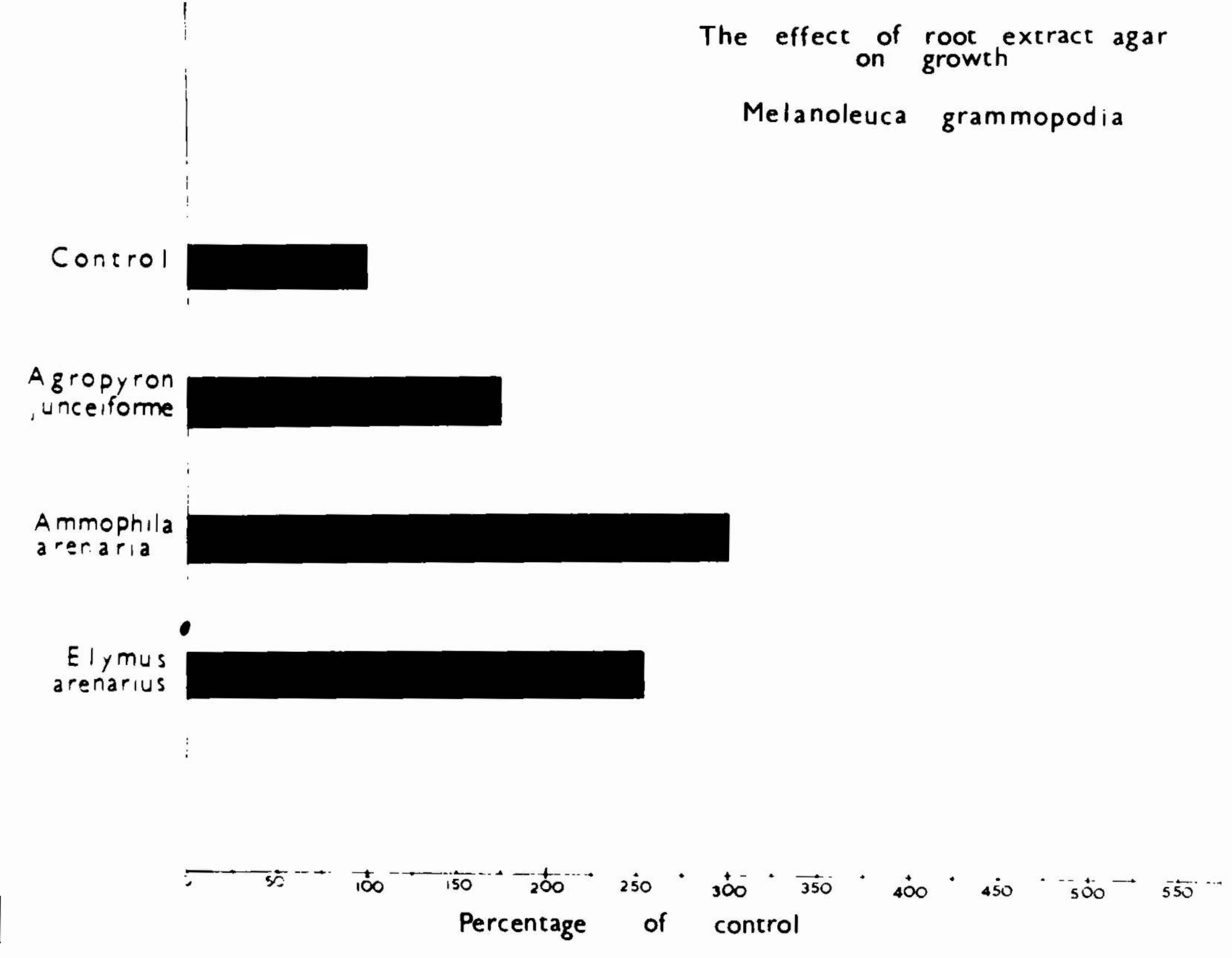

The effect of root extract agar on growth
Melanoleuca grammopodia
Control
Agropyron junceiforme
Ammophila arenaria
Elymus arenarius
0
50
100
150
200
250
300
350
400
450
500
550
Percentage of control

PLATE 67

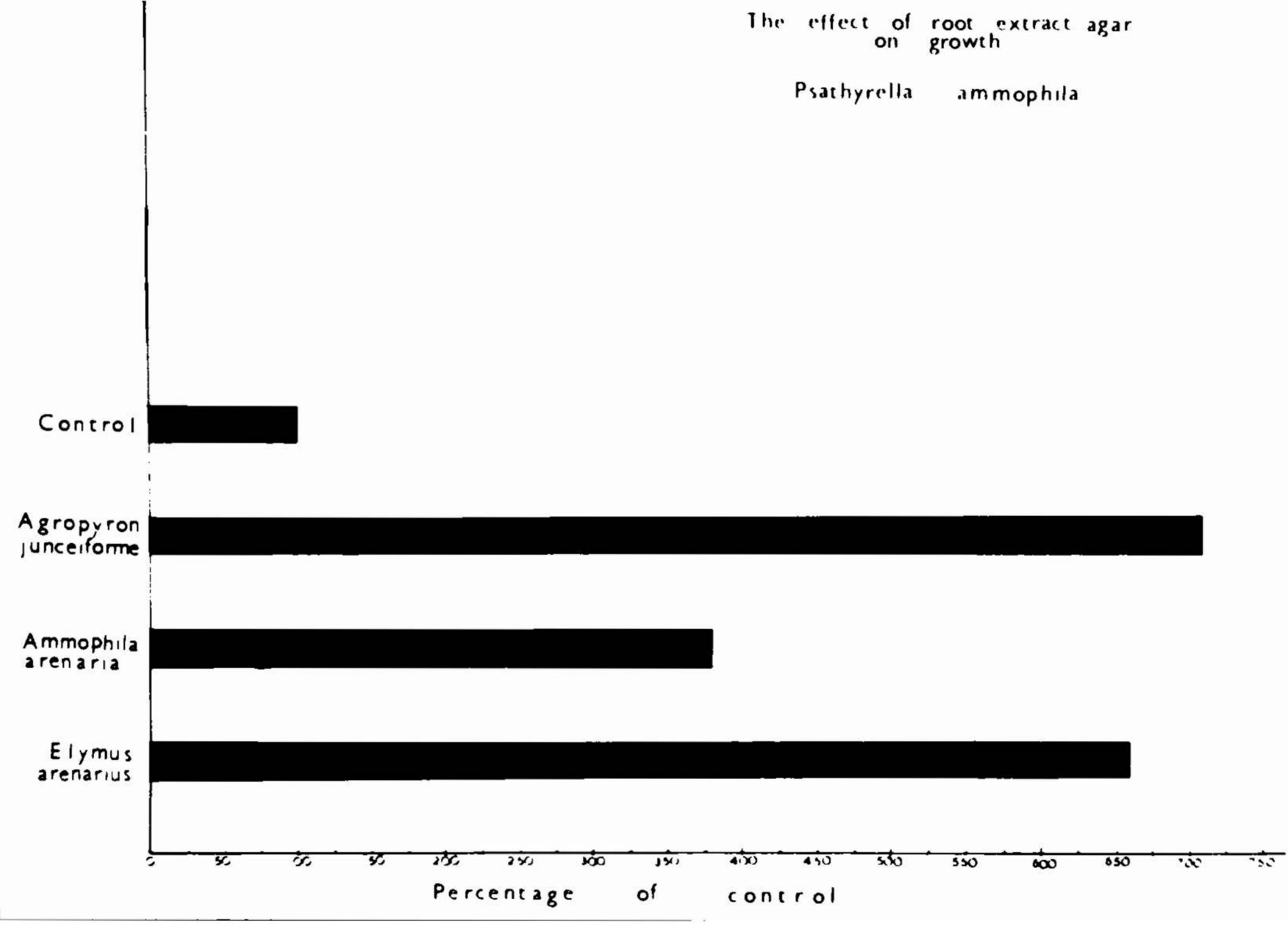

The effect of root extract agar on growth
Psathyrella ammophila
Control
Agropyron junceiforme
Ammophila arenaria
Elymus arenarius
Percentage of control

PLATE 68

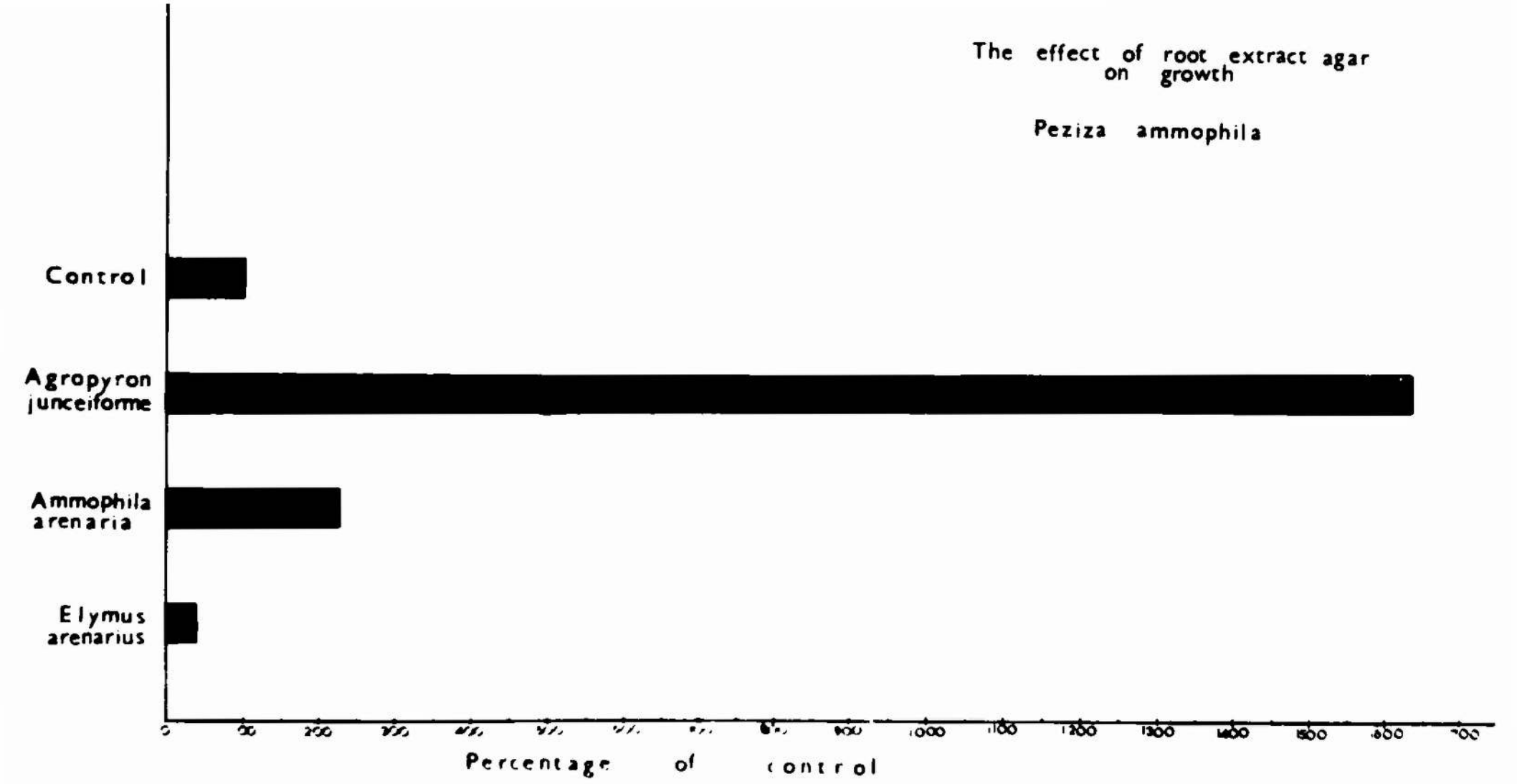

The effect of root extract agar on growth
Peziza ammophila
Control
Agropyron junceiforme
Ammophila arenaria
Elymus arenarius
Percentage of control

PLATE 69

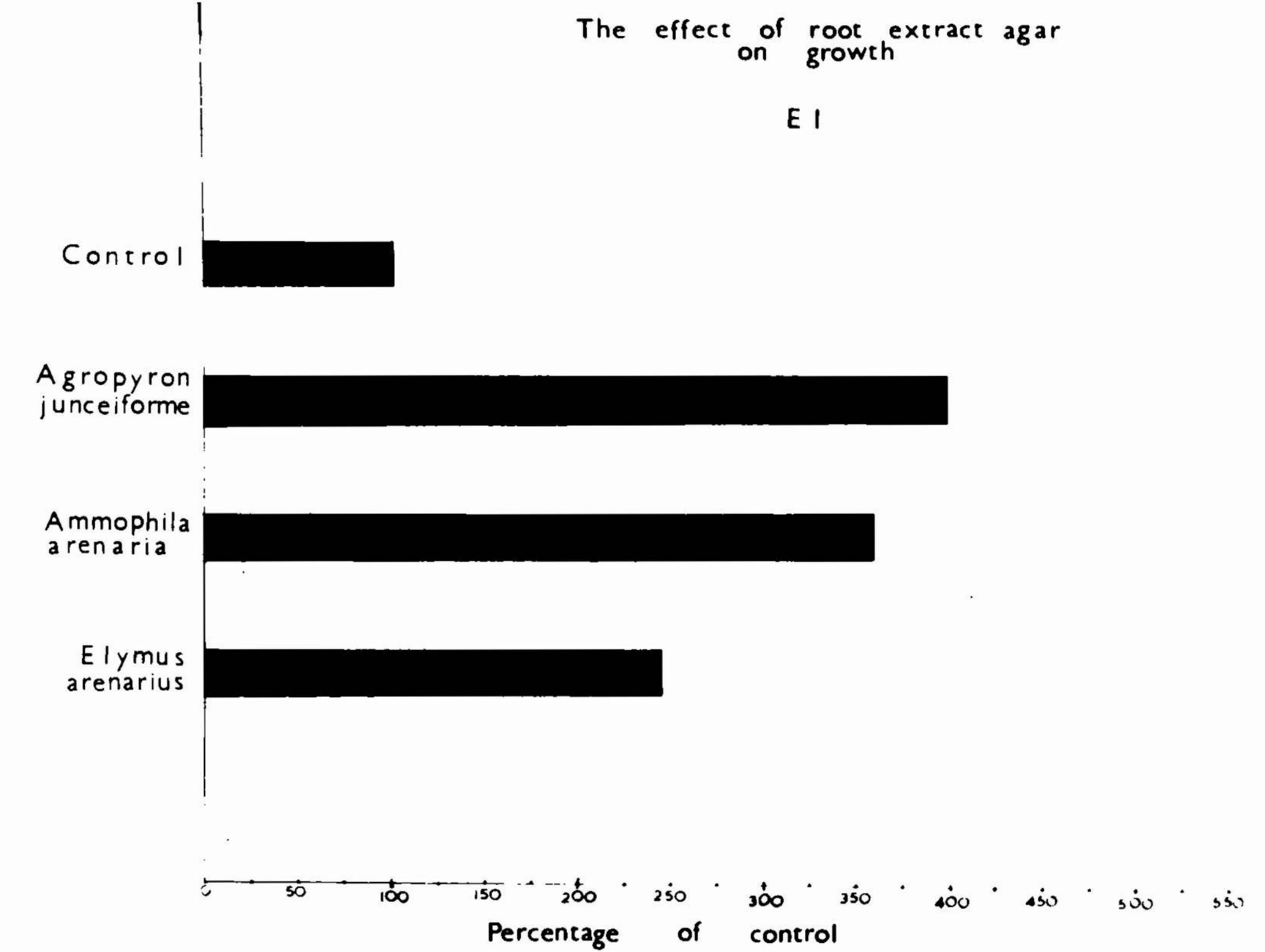

The effect of root extract agar on growth
E 1
Control
Agropyron junceiforme
Ammophila arenaria
Elymus arenarius
0
50
100
150
200
250
300
350
400
450
500
550
Percentage of control

PLATE 70

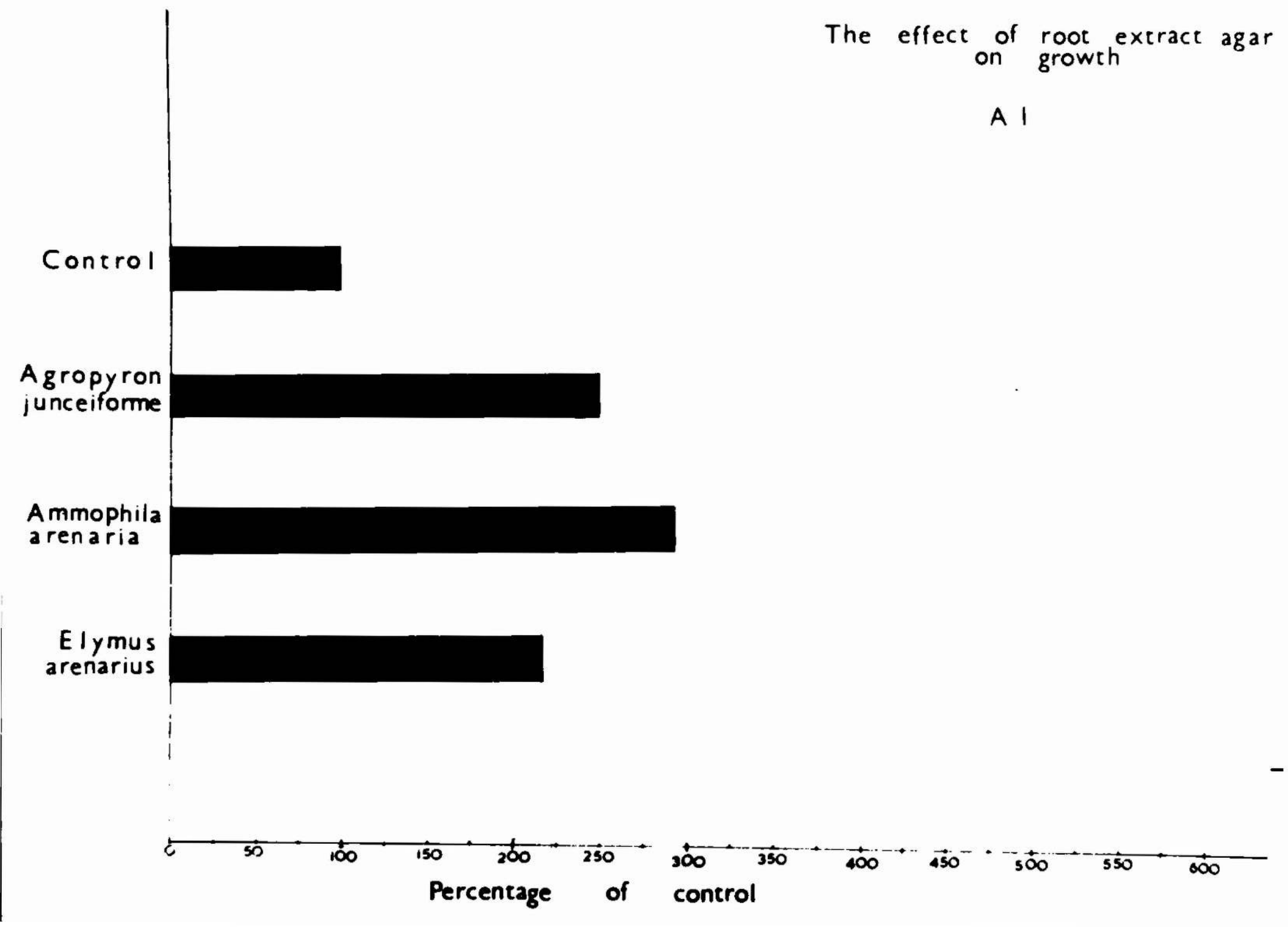

The effect of root extract agar on growth
A 1
Control
Agropyron junceiforme
Ammophila arenaria
Elymus arenarius
0
50
100
150
200
250
300
350
400
450
500
550
600
Percentage of control

PLATE 71

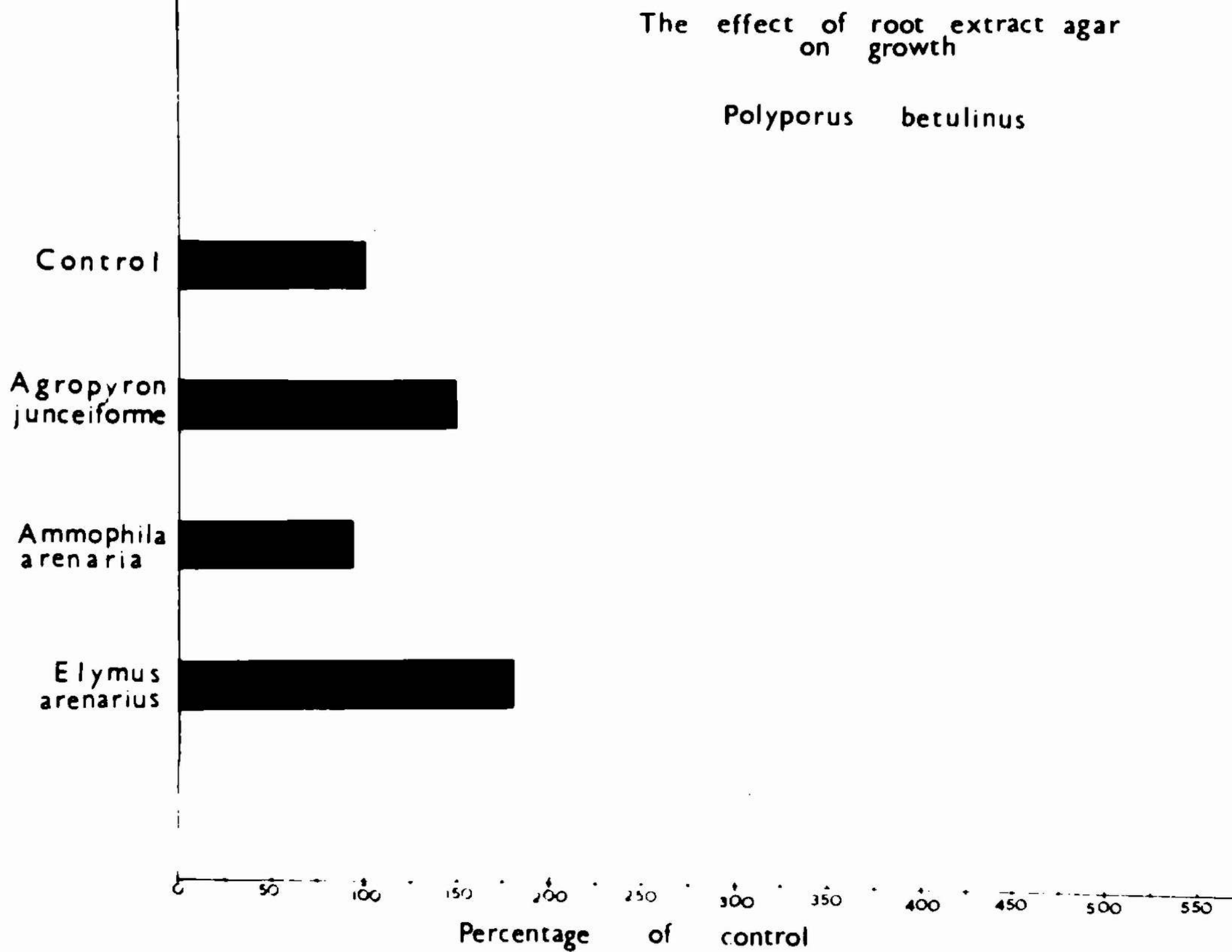
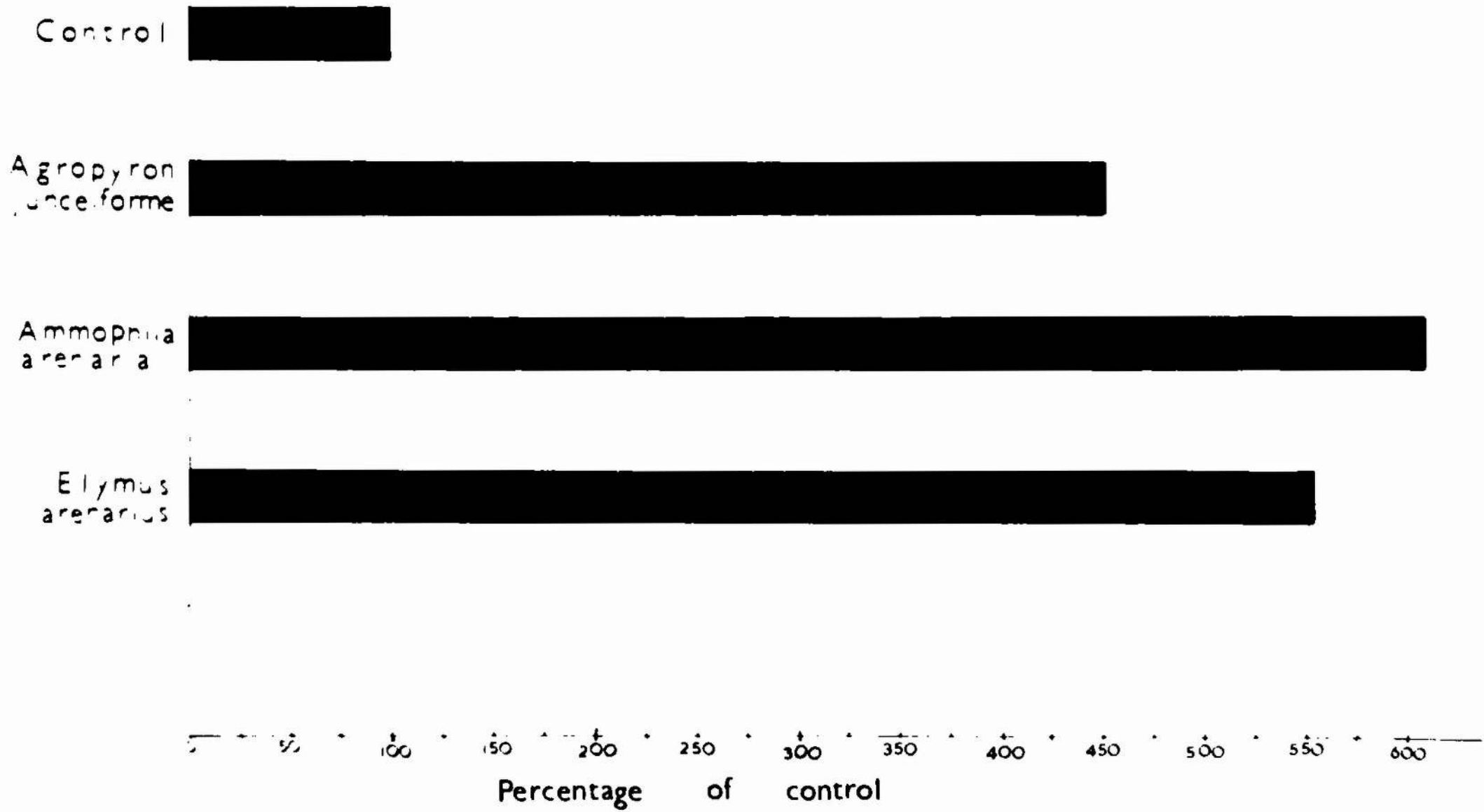

The effect of root extract agar on growth
Polyporus betulinus
Control
Agropyron junceiforme
Ammophila arenaria
Elymus arenarius
0
50
100
150
200
250
300
350
400
450
500
550
Percentage of control

PLATE 72

The effect of root extract agar on growth

Panaeolus semi ovatus



would be unwise to jump to the conclusion that Agropyron stimulated its growth by 16 fold whereas Elymus inhibited it.

The root extracts had a stimulating effect on the growth of E 1. The results, however, show that the least stimulation was obtained from Elymus extract, the root from which this fungus was isolated and that the greatest stimulation was obtained on the extract from Agropyron. The extract which caused greatest stimulation of A 1 was that of Ammophila. This is interesting since this fungus was isolated from the roots of Ammophila. The stimulation obtained by A 1 from the other two extracts was very similar.

The results of the two test fungi, Panaeolus and Polyporus show some amount of stimulation especially in the case of Panaeolus and except in the case of Polyporus on Ammophila extract.

Discussion

Many investigators have shown that extracts from plant tissues have stimulatory effects on the growth of fungi in culture. Smart (1937) found that extracts from pea, butter bean, fallen oak leaves, fallen pine needles, corn, rotten pine wood, hay infusion, hemp fibres etc. stimulated the germination of spores of Myxomycetes. Osborne and Thrower (1964) reported that methanolic and aqueous extracts of wood of Eucalyptus marginata and E. diversicolor stimulated the growth of Polyporus zonalis and P. dryadeus.

The results obtained in the present investigation show that, if the greatest stimulation for each fungus is taken into account, then Agropyron extract stimulated

the growth of Conocybe, Peziza, Psathyrella and E 1; Ammophila extract produced stimulation in Melanoleuca, A 1 and Panaeolus whereas Elymus extract stimulated Polyporus. Inhibitory effects were observed only twice: Elymus inhibited the growth of Peziza, and Ammophila had an inhibitory effect on Polyporus.

Since Polyporus, a wood-rotting fungus, and Panaeolus a coprophilous fungus, were both stimulated by the root extracts of the sand-dune grasses, then it is clearly demonstrated that the stimulation is a very generalized one and not specific to the fungi confined to the sand-dunes. This is not an unusual phenomenon since Melin (1954) found that the roots of many plants other than potential hosts produced exudates which stimulated mycorrhizal fungi. In the same year, Melin and Das (1954) used Boletus elegans, B. variegatus, Russula xerampelina and Rhizopogon roseolus as test fungi and found that they were stimulated by exudates of roots of several herbaceous angiosperms. Although the current experiment is not dealing with root exudates, the analogy is still there.

The stimulant which was present in the root extracts is both water soluble and heat resistant and probably the conditions under which the experiment was carried out were directly responsible for the trend that the results have taken, because although the results suggest that the higher fungi should be associated with A. juncoiforme more than with E. arenarius, this is not the case on the sand-dune in Tentsmuir.

5d Production of Root Exudates and their effects on the Growth of the Isolates.

This experiment was conducted with a view to ascertaining whether the grasses exuded any substance through their roots which might have beneficial or deleterious effects on the growth of the fungi. No attempt was made to analyse the liquid to find out what substances, if any, were exuded.

Method

The method employed here was a modification of that used by Sulochana (1962a and b) in her investigation of amino acids and vitamins in root exudates of cotton.

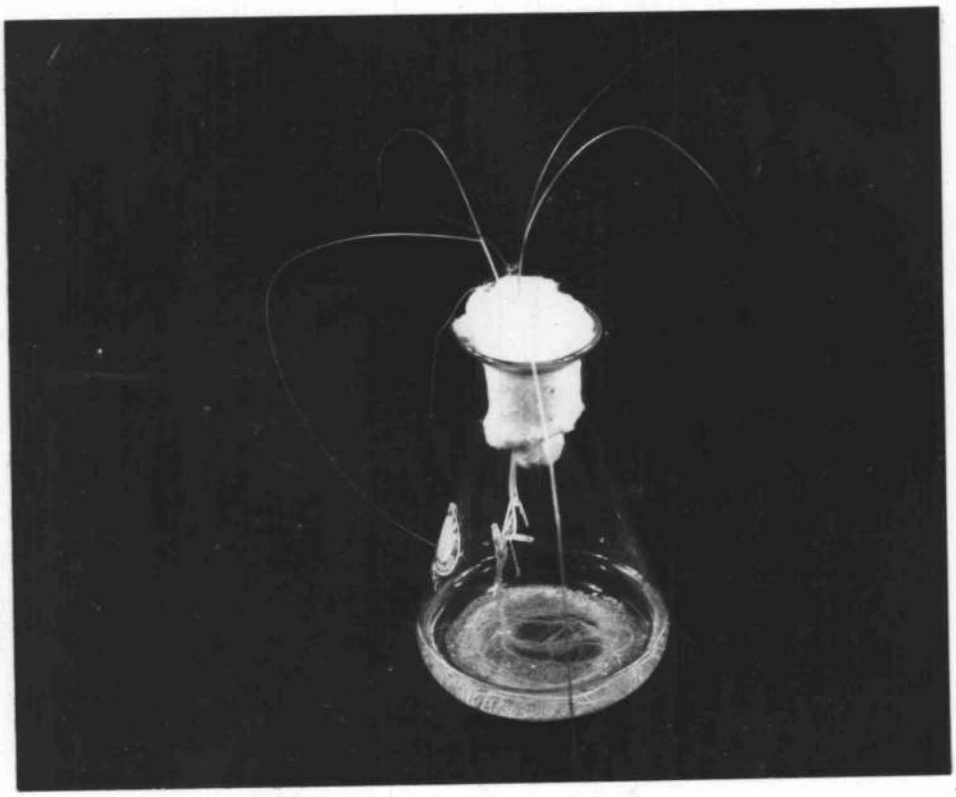
The seedlings were grown as shown in Method, 2(b)(v). When they had grown to an average height of 5cm above the sand surface they were removed and transferred to 100ml Erlenmeyer flasks which contained 30ml distilled water and 3 grams sand and had been previously sterilized by autoclaving for thirty minutes at 20 lbs. pressure per square inch.

Great care was exercised in removing the seedlings from the sand to prevent the delicate roots from breaking. After removal, the roots were washed in sterilized distilled water and three or four seedlings were put in each flask so that the root systems were submerged but not the stems (Plate 73). Each flask was then wrapped in aluminium foil to discourage algal growth and was put in a glass house at a temperature of about 60° F. A total of 24 flasks per plant species was set up.

After three weeks, 12 flasks per species were removed; the other 12 remained for six weeks. At the end of these periods, the remaining liquid was poured off into sterilized measuring cylinders, one for each

PLATE 73

Three-week-old plants of *A. junceiforme* in sand-water culture.



species of grass. The seedlings were transferred to freshly prepared flasks and allowed to grow for a further nine and six weeks respectively, by the end of which time the plants were twelve weeks old, and the liquid was again used.

After thorough mixing, the liquid was dispensed in 15ml quantities in previously sterilized 100ml Erlenmeyer flasks and to each was also added 10ml of the liquid which was used as a control. For the control, 30ml distilled water and 3 grams sand were put in a flask, sterilized as the others and kept under similar conditions. Twenty-five ml of the supernatant liquid were poured in sterilized 100ml flasks.

All the flasks were then inoculated with 5 x 5 x c.2mm inoculants which were taken from cultures of C. dunensis, Psathyrella ammophila, M. grammopodia, Polyporus betulinus, Panaeolus semi-ovatus, E 1 and A 1. These fungi had been actively growing on malt extract agar. The inoculated flasks were then incubated at 23°C for eight weeks after which time they were oven dried at 50°C to constant weight and their dry weights determined (Table 17).

Results

Growth of the fungi under all conditions was very poor. This is obvious when Table 17 is examined. In some cases there was no visible mycelium and so the bulk of the weights was due to the agar of the inocula and not to the mycelium. Although equal sized inocula were used, they were taken from different cultures at the three different times i.e. at three, six and twelve weeks. Consequently, growth cannot justifiably be

TABLE 17

Growth of Fungi on Root Exudates

Dry weights in mg. Average of 2 replicates.

Fungi		3 weeks			6 weeks			12 weeks		
	Control	Agropyron	Ammophila	Elymus	Agropyron	Ammophila	Elymus	Agropyron	Ammophila	Elymus
C. dunensis	16.9	2.9	6.1	5.5	1.9	5.2	4.4	4.9	5.9	4.1
Psathyrella ammophila	12.2	3.0	2.2	2.4	2.5	3.9	4.1	3.9	5.0	5.4
M. grammopodia	10.0	2.1	3.2	3.2	2.1	3.8	2.9	3.8	4.2	4.9
Polyporus betulinus	11.1	3.5	1.5	1.3	2.4	3.4	3.4	2.3	3.2	3.6
Panaeolus semi-ovatus	10.5	4.4	1.4	1.5	2.0	6.0	4.0	6.3	5.7	4.7
E 1	10.1	1.6	4.5	5.2	2.1	4.4	3.6	3.1	4.4	4.6
A 1	12.4	2.0	4.7	5.7	2.2	4.6	4.5	4.5	3.7	3.9

compared at the different grass ages since the thickness of the agar inocula would greatly affect the weight. Comparison, however, can be made with the interaction of each of the three grasses of similar ages on each of the fungi.

From the dry weights it could be seen that growth of the fungi was different with the three grasses and so statistical analyses were carried out. The design used was a split plot design in which the nine main plots consisted of a single replicate of a 3^2 fractional design with grasses and ages as the two factors. Each main plot was then subdivided into four subplots consisting of a double replicate for seven fungi. From a statistical point of view the design of the experiment was unsatisfactory since no error was available for testing the main plot analysis. However, the following information was obtained:-

(1) No obvious pattern arose with respect to the age of the grasses.

(2) Most of the differences were highly significant at the 1% level of significance and very little was found that was insignificant.

(3) The means for A. arenaria and E. arenarius were in close proximity and were significantly higher than the means of A. junceiforme.

(4) Since A. arenaria and E. arenarius seemed to be so similar, an analysis was done to see how they reacted to the different fungi. The results were still highly significant, that is, the grasses reacted differently with the different fungi.

(5) From the results of (4) above, it was found that the difference between A. arenaria and E. arenarius was mainly due to their interaction with C. dunensis and P. semi-ovatus. These two fungi had the highest means almost all the time.

(6) A t-test showed that the interaction of C. dunensis and P. semi-ovatus with the two grasses was significantly different.

Discussion

From the results of this experiment, it seems likely that the sand-dune grasses produce substances which stimulate the fungi differently. The concentration of the stimulants was undoubtedly very small. This could be due to the age of the plants and/or the conditions of the experiment. Apparently, the stimulant was not specific in its effect, as the two test fungi did not produce different growth patterns from the obligate sand-dune ones.

An obvious deficiency of this experiment is the conditions under which the seedlings were grown. The conditions were unnatural in many respects, for example, the root systems were submerged and the medium was sterile. To obtain root exudates, it is necessary to maintain normal plant growth under aseptic conditions. Otherwise, it would be impossible to determine the source of the stimulant. The plants would probably behave differently if they were grown in sterile sand, but it would be difficult to separate the exudate from the sand especially if the quantity was very small. Under these unnatural conditions, root excretions which

are due to damaged roots or sloughing off of cells would be at a minimum and any stimulation would be due to true exudations.

The use of distilled water in this experiment and not nutrient solution probably explains the low stimulation. After the initial period of growth in which the nutrients present in the medium were used up, the supply of nutrients in one form or another probably became limiting and controlled the amount of exudation which took place. But it was thought that any addition would result in concentrations of certain mineral elements high enough to be unfavourable to plant growth. Sulochana (1962) grew cotton plants under similar conditions and found that they exuded amino acids and B-vitamins. In the present experiment, it was thought that if they were root inhabiting or rhizosphere fungi, the roots would provide them with the substances necessary for growth and so no further additions would be needed. But the fact that the dry weights of the fungi on the control medium were so much higher than those on the other solutions suggests that the plants had removed certain elements which were essential for optimum growth of the fungi.

Katznelson, Rouatt and Payne (1954, 1955) found that when plants in sandy soils were dried and re-moistened, appreciable amounts of amino acids and reducing compounds were liberated. This suggestion implies that the conditions of the present experiment were not conducive to root exudation. However, it is not unlikely that in the sand-dune where there is frequent drying and remoistening this phenomenon also occurs and provides the fungi with food supply.

Roots are capable of exuding nitrogenous compounds especially amino acids (Katznelson et al, 1954, 1955; Pearson and Parkinson, 1961; Sulochana, 1962a; Vančura and Hovadik, 1965). Vitamins and other growth stimulating substances are also important constituents of root exudates (Hansteen-Cranner, 1922; West, 1939; Lundegårdh and Stenlid, 1944; Melin, 1954; Melin and Rama Das, 1954; Rovira, 1956b; Rovira and Harris, 1961 and Sulochana, 1962). Lundegårdh and Stenlid (1944), Rovira (1956a) and Vancura (1964) are among the many workers who found that carbohydrates, especially reducing substances, are exuded from roots.

De Silva and Wood (1964) have shown that appreciable stimulation was obtained only when the exudates of cabbage and lettuce were collected in the first seven days. Hansteen-Cranner (1922), West (1939), Rovira (1956a), Pearson and Parkinson (1961) and Vančura (1964) seem to agree with their finding.

Rovira (1956a) reported that for the first three weeks of growth of peas and oats exudation formed the bulk of the material coming from the roots and that only a slight amount was due to autolysis but that as the plants developed, true root exudations became less important and excretions due to autolysis became increasingly important.

In the sand-dune, propagation is not usually by seeds producing seedlings and so root exudation may not be as important to the growth of the fungi as excretion resulting from autolysis and the sloughing off of cells. Root exudation and root excretion are natural processes in plants and micro-organisms are dependent on them for their nitrogenous and carbonaceous substances as well

as the growth stimulating substances. In the sand-dune where the grasses are in their natural environment they are no doubt exuding nutrients which contribute to the optimum growth of the fungi in their vicinity.

SECTION 6

6a General Discussion and Conclusion

The exact ecological niche which the higher sand-dune fungi occupy cannot definitely be determined from the experimental data which are available at present. There is evidence against their being either soil inhabiting fungi or obligate parasites and evidence for and against their being saprophytic fungi or root inhabiting fungi which consists of mycorrhizas and parasites.

They have been relatively easily isolated on synthetic media, a characteristic which implies that they could not be obligate parasites in the strict nutritional sense. Their growth in culture was on the whole very slow and they were very easily overgrown by known soil inhabiting fungi e.g. species of Penicillium and Aspergillus. Furthermore, there is evidence to show that they do not possess the other characteristics of soil inhabiting fungi i.e. (i) freely growing through unsterilized soil, (ii) being able to colonize only dead plant material and (iii) distribution in the soil being general (Garrett, 1956).

Andersson (1950) and Wallace (1954) surmised that the larger sand-dune fungi might form mycorrhizal partnership with the living roots of the grasses. So far, there is no evidence confirming this. Nicolson (1959, 1960) listed the fungi which he observed in the roots of the sand-dune grasses as vesicular-arbuscular endophytes, Rhizoctonia type hyphae, brown septate hyphae and Chytridiaceous fungi, but he made no mention of any Basidiomycete being there as well. Whether they are

present in those plants in Tentsmuir and not in those at Gibraltar Point is not known.

Three apparently different unidentified Basidiomycetes (E 1, A 1 and A 2) have been isolated from roots of A. arenaria and E. arenarius during the course of the present investigation, and microscopic observation reveals their relatively common occurrence in mature roots. Their presence in mature tissues does not necessarily make them saprophytes as the roots could have been infected while still alive. The cultural and physiological characteristics of these isolates do not resemble those of the large fungi whose fruit bodies appear annually in the area, but the bulbous nature of the hyphae of A 1, E 1, C. dunensis and M. grammopodia might be significant.

The ratio of the amount of nitrogen and phosphorus available to the plant to the amount of soluble carbohydrate material in the root has been shown to regulate the amount of mycorrhizal roots formed, (Björkman, 1949; Handley and Saxinders, 1962; Richards and Wilson, 1963; Richards, 1965). High carbohydrate concentration and low nitrogen and phosphorus encourage the production of mycorrhizal association. Failure of the fungi to form mycorrhiza in culture could be due to an unfavourable proportion of these elements. It was also shown that mycorrhizal roots of beech were able to absorb large amounts of phosphorus and actively transport it into the roots (Harley and McCready, 1952; Harley and Brierley, 1954, 1955). Gerdemann (1964) found experimentally that the function of mycorrhiza was to make phosphorus available, increase its absorption or assist

the host in its metabolism. Ternetz, Duggar and Davis (Harley, 1959) have obtained evidence of a slow but highly efficient fixation of atmospheric nitrogen by Phoma radicis. Hassouna and Wareing (1965) have inferred that rhizosphere bacteria are playing an important role in nitrogen fixation in the roots of A. arenaria in sand-dunes. Indeed, fungi forming mycorrhizal relationships which are able to assist in the availability of phosphorus and nitrogen would be invaluable to plants which live under conditions of very low supply of these elements.

It is also a well established fact that fungi obtain their soluble carbohydrates, vitamins and other growth factors from the higher plants with which they form mycorrhizae (Melin, 1946; Björkman, 1949; Handley and Saunders, 1962). The suggestion was made that they fail to produce sporophores in the absence of their host because of their inability to synthesize these substances (Melin, 1946; Romell, 1938, 1939).

It was experimentally shown that all the sand-dune fungi under investigation grew better when thiamine was added to a medium containing glucose and mineral salts, and that M. grammopodia and Peziza ammophila also grew better when biotin was added to the medium. Pyridoxine, as well as thiamine, stimulated the growth of C. dunensis and A 1. All the fungi grew poorly on the basal medium, therefore, they are probably able to synthesize a certain amount of these vitamins but not enough to support optimum growth. The grasses could conceivably supply them with adequate amounts perhaps through exudation.

Björkman (1949) cited Thesleff and Romell as being two of the many observers who found that sporophores of mycorrhizal-forming fungi occurred in association with full grown trees and not in the vicinity of seedlings. Rayner (1927) remarked that a certain internal physiological condition must be reached by the seedling before mycorrhizal infection can take place. A review of root exudation shows that carbonaceous, nitrogenous, phosphotides and other physiologically active substances are exuded from roots in their initial growing phase (Hansteen-Cranner 1922; West, 1939; Rovira, 1956a, b, c; de Silva and Wood, 1964 and Vančura, 1964) or predominantly from the root tips (Pearson and Parkinson, 1961; Schroth and Hildebrand, 1964). However, Vančura and Hovadik (1965) have shown that there are possible changes in the nature of root excretions during the development of the plant. Rovira (1956a) suggested that as plants develop, true root excretions are probably not as important in influencing the rhizosphere population as is the cast off cell material. Decortication also causes leaking out of materials from the interior of roots. Probably the fully mature plants are able to excrete the kind or kinds of substances specifically required for mycorrhizal development and sporophore production. This would explain the negative results obtained in synthetic mycorrhizal formation of the sand-dune fungi with the eight months old grasses.

Tree mycorrhizal fungi develop in a characteristic manner between the cortical cells of the roots after the middle lamellae have decomposed. This suggests that

pectins are readily utilized by the fungi. Melin (1948) in his studies of tree mycorrhiza showed that the fungi will grow on pectic substances. From viscosimetric determinations it was found that C. dunensis, M. grammopodia and E 1 were able to produce large quantities of extracellular pectinolytic enzyme while Psathyrella ammophila, Peziza ammophila and A 1 produced only negligible quantities. Nevertheless, in nature all the sand-dune fungi might be producing this enzyme which would be a necessity in mycorrhizal development.

Melin (1922, 1948) pointed out that mycorrhizal fungi on the whole make better use of organic than inorganic nitrogen. Norkrans (1950) also reported that members of Tricholomaceae - a predominantly mycorrhizal forming group - were able to utilize ammonium and organic nitrogen while nitrate nitrogen could not be assimilated. Experimental evidence has been brought forward that all except one of the higher sand-dune fungi utilized organic nitrogen much better than ammonium or nitrate nitrogen. Of the seven sources of nitrogen supplied to them (Table 3) they utilized asparagine and glycine best. Psathyrella ammophila was able to assimilate ammonium nitrogen better than organic or nitrate nitrogen.

Although there is much evidence to suggest that the higher sand-dune fungi are capable of forming mycorrhiza, there is still no conclusive evidence that they do. They were unable to form synthetic mycorrhiza, but as has already been discussed this might be a result of unfavourable environmental conditions in the experiments and is not convincing proof of their inability to form such an association with the higher plants in nature.

Perhaps under the conditions of the experiment, their nutritional requirements were satisfied and they did not need to form an intimate relationship with the grasses. However, their constant appearance among the grasses in the sand-dunes is strong evidence of environmental specificity.

On the other hand, it can be argued that they are saprophytes or rhizosphere inhabiting fungi. Their ability to decompose cellulose and lignin is strong evidence of their being saprophytes, although Lindeberg (1948) showed that Boletus subtomentosus and Lactarius deliciosus, both mycorrhiza formers, gave strong oxidative reactions on tannic and gallic acid agars. Melin (1948) also inferred that although cellulose and lignin do not seem to be utilized as sole carbon sources by mycorrhizal fungi, they can form cellulose splitting enzymes which enable them to pierce the cell walls and invade the cells. The inability of the sand-dune fungi to utilize straw of E. arenarius in flasks does not eliminate the possibility of their doing so in nature. C. dunensis and Psathyrella ammophila have fruited under conditions which also suggest that they are saprophytes and the presence of hyphae of C. dunensis, M. graminopodia and Psathyrella ammophila in the crown regions of old roots only is also strong evidence of their saprophytic capacities.

Their hyphae were able to enter roots in which there was no obvious sign of previous infection and the reason for the low infection might be due to environmental conditions and not to their inability to infect living tissues. The literature shows high correlation between production of pectinolytic and cellulolytic

enzymes and pathogenicity and saprogenicity (Husain and Rich, 1958; Husain and Dimond, 1960; Winstead and McCombs, 1961; Barker and Walker, 1962; Leal and Villanueva, 1962; Spaldings, 1963; Keen and Horton (1966). Therefore, the ability of the higher sand-dune fungi to produce extracellular pectinolytic and cellulolytic enzymes makes them potential saprophytes as well as potential pathogens. Root inhabiting fungi are restricted in their saprophytic phase because of the competition of other soil micro-organisms. The higher sand-dune fungi grow very slowly in culture and if they behave similarly in nature they would be very poor saprophytic competitors.

Therefore, since (i) they are suppressed by saprophytes on agar plates; (ii) they do not grow through unsterilized soil in the absence of food base (they have never appeared on soil dilution plates or any such isolation technique); (iii) they are capable of infecting living tissues and (iv) their distribution in the soil depends on the presence of host plants and is, therefore, local (they occur only in sand-dunes with the obligate sand-dune grasses, A. arenaria, E. arenarius and A. junceiforme) and since these are the characteristics of Garrett's root inhabiting fungi (Garrett, 1956), it is suggested that some of them, if not all, can be classified with this group of fungi and that further experimental evidence is necessary before they can be called mycorrhizal or parasitic.

The writer regrets that she has not been able to give more precise answers to the nutritional relationship between the higher fungi and these sand-dune grasses, but hopes she has paved the way for further research.

6b Summary

The object of this work has been to investigate the nutritional status of the higher fungi on the sand-dune at Tentsmuir, with special emphasis on mycorrhizal formation.

(i) The higher fungi comprised (a) Conocybe dunensis, Wallace; (b) Melanoleuca grammopodia, (Fries ex Bulliard) Pat. [= Tricholoma melaleucum (Pers. ex Fries) Kummer]; (c) Psathyrella ammophila (Dur. et Lév.) Orton; [= Psilocybe ammophila (Dur. et Lév.) Gillet]; (d) Peziza ammophila (Dur. et Montagne) Cooke [= Geopyxis ammophila (Dur. et Montagne) Cooke]. The grasses on the fore dune were Ammophila arenaria, L., Agropyron junceiforme, A. and D. Love and Elymus arenarius, L.

(ii) The fungi were isolated on malt extract agar using gills of the Agarics and hymenium of the Discomycete (Section 2(b)(iv).

(iii) Two unidentified Basidiomycetes (A1 and A2) have been isolated from roots of Ammophila arenaria and one (E1) from roots of Elymus arenarius (Section 2(b)(iii). A 1 and E 1 were subjected to the same treatments as the known fungi.

(iv) Their vitamin requirements have been investigated with regard to thiamine, biotin and pyridoxine. All the fungi grew better when thiamine was added; biotin also stimulated the growth of M. grammopodia and Peziza ammophila while C. dunensis and A 1 needed pyridoxine also for optimum growth (Section 4a).

(v) Ammonium nitrogen was assimilated by Psathyrella ammophila. All the others utilized organic nitrogen

especially asparagine and glycine. With the exception of Psathyrella ammophila and Peziza ammophila they grew very slowly on nitrate nitrogen (Table 4).

(vi) Under the experimental conditions, C. dunensis, M. grammopodia and E 1 produced large quantities of extracellular pectinolytic enzyme (possibly polygalacturonase) while the others produced little or none at all.

(vii) Tricholoma nudum and Panaeolus semi-ovatus known cellulose attackers which were used as test organisms decomposed carboxymethylcellulose but not ballmilled cellulose. E 1, A 1 and C. dunensis also decomposed carboxymethylcellulose but very slowly. The others did not produce cellulolytic enzymes in vitro (Plates 30-40).

(viii) Psathyrella ammophila, C. dunensis, M. grammopodia, E 1 and A 1 produced varied quantities of polyphenol oxidase on tannic and gallic acids suggesting their ability to utilize lignin as a carbon source (Plates 43-48).

(ix) Spores of C. dunensis germinated readily in tap water; those of Psathyrella ammophila on modified Lange's medium and Oxoid Nutrient agar (Cm3); those of Peziza ammophila on malt extract agar but better on Lange's modified medium and those of M. grammopodia failed to germinate under all the conditions given (Section 4e).

(x) Psathyrella ammophila produced fruit bodies in pots with A. juncoiforme only. C. dunensis fruited readily in pots with and without each of the grasses. A study has been made of the possible effects of the grasses on the time and frequency of sporophore production (Section 5 (a)(ii)(a)).

(xi) The effects of the fungi on the dry weight of aerial parts of the plants were not conclusively demonstrated (Section 5(a)(ii)(b)).

(xii) An attempt was made to explain negative results in the formation of synthetic mycorrhiza (Section 5(a)(ii)(c)).

(xiii) Heat resistant water soluble stimulants were present in extracts of the roots of all the grasses. That of A. junceiforme stimulated the growth of C. dunensis, Psathyrella ammophila, Peziza ammophila and the isolate from Elymus roots. That of A. arenaria stimulated the growth of M. grammopodia and the isolate from roots of A. arenaria. All the fungi except Peziza ammophila showed some stimulation by the extract of Elymus roots.

(xiv) A study has been made of the production of root exudates and their possible effects on the growth of the fungus isolates.

(xv) A preliminary survey was made of the incidence of infection of A. arenaria, A. junceiforme and E. arenarius by Basidiomycetes in the field in May and September.

SECTION 7

BIBLIOGRAPHY

Andersson, O. 1953. Larger fungi on sandy grass heaths and sand-dunes in Scandinavia. Botaniska Notiser, Supplement Vol. 2:2.

Apinis, A.E. 1965. A new thermophilous Coprinus species from coastal grassland. Trans. Brit. mycol. Soc., 48:653-656.

Bain, Henry 1937. Production of synthetic mycorrhiza in Cranberry. J. agric. Res., 55:811-835.

Barker, K.R. and Walker, J.C. 1962. Relationship of pectolytic and cellulytic enzyme production by strains of Pellicularia filamentosa to their pathogenicity. Phytopathology, 52:1119-1125.

Barnett, H. L. and Lilly, V.G. 1948. The interrelated effects of vitamins, temperature and pH upon vegetative growth of Sclerotinia camelliae. Amer. J. Bot., 35:297-302.

Bateman, D.F. 1964. Cellulase and the Rhizoctonia disease of bean. Phytopathology, 54:1372-1377.

Bateman, D.F. and Millar, R.L. 1966. Pectic enzymes in tissue degradation. Ann. Rev. of Phytopathology, 4:119-146.

Bent, K.J. and Morton, A.G. 1963. Formation and nature of swollen hyphae in Penicillium and related fungi. Trans. Brit. mycol. Soc., 46:401-408.

Björkman, Erik 1949. The ecological significance of the ectotrophic mycorrhizal association in forest trees. Svensk bot. Tidskr., 43:223-262.

Brown, J.C. 1958. Soil fungi of some British sand-dunes in relation to soil type and succession. J. Ecol., 46:641-664.

Brown, W. 1936. The physiology of host-parasite relations. Bot. Rev., 2:236-281.

Chesters, C.G.C. 1960. Certain problems associated with the decomposition of soil organic matter by fungi. The Ecology of Soil fungi. An international symposium. Liverpool University Press.

Chesters, C.G.C. and Parkinson, D. 1959. On the distribution of fungi in the rhizosphere of oats. Plant and Soil, 11:145-156.

Cochrane, V.W. 1958. Physiology of Fungi. Wiley & Sons, Inc. N.Y. Chapman & Hall, Ltd., London.

Davidson, R.W., Campbell, W.A. and Blaisdell, D.J. 1938. Differentiation of wood decaying fungi by their reactions on gallic or tannic acid medium. J. agric. Res., 57:683-695.

De Bary, A. 1887. Comparative morphology and biology of the Fungi, Mycetozoa and Bacteria. Clarendon Press, Oxford. English translation.

Dennis, R.W.G. 1960. British Cup Fungi and their allies. Ray Society London.

Dennis, R.W.G., Orton, P.D. and Hora, F.B. 1960. New check list of British Agarics and Boleti. Trans. Brit. mycol. Soc., 43 Supplement.

De Silva, R.L. and Wood, R.K.S. 1964. Infection of plants by Corticium solani and C. praticola. Trans. Brit. mycol. Soc., 47:15-24.

Dobbs, C.F. and Gash, M.J. 1965. Microbial and residual mycostasis in soils. Nature, Lond., 207:1354-1356.

Dos Santos, N.F. 1963. Nitrogen utilization by 4 isolates of Armillaria mellea. Trans. Brit. mycol. Soc., 46:281-284.

Edgington, L.V., Corden, M.E. and Dimond, A.E. 1961. The role of pectic substances in chemically induced resistance to Fusarium wilt of tomato. Phytopathology, 51:179-182.

Elarosi, H. 1958. Fungal associations. (iii) The role of pectic enzymes on the synergistic relation between Rhizoctonia solani Kühn and Fusarium solani Snyder and Hansen in the rotting of potato tubers. Ann. Bot., London, 22:399-416.

Fries, L. 1955. Physiology of Coprinus. (i) Growth substance, nitrogen and carbon requirements. Svensk bot. Tidskr., 49:475-535.

Fries, N. 1943. The effect of adermin, aneurin and biotin on the growth of various Ascomycetes. (English Summary). Symb. bot. Upsaliens., 7(2):67-73.

Fries, N. 1966. in The fungus spore. Butterworths, London.

Gadgil, P.D. 1965. Distribution of fungi on living roots of certain Graminaae and the effects of root decomposition on soil structure. Plant and Soil, 22:239-259.

Garrett, S.D. 1937. Soil conditicns and the take all disease of wheat (ii) The relation between soil reaction and soil aeration. Ann. appl. Biol., 24:747-751.

Garrett, S.D. 1953. Rhizomorph behaviour in Armillaria mellea (Vahl.) Quél. (i) Factors controlling rhizomorph initiation by A. mellea in culture. Ann. Bot., Lond., 17:63-79.

Garrett, S.D. 1956. Biology of Root Infecting Fungi. University Press Cambridge.

Garrett, S.D. 1963. A comparison of cellulose decomposing ability in 5 fungi causing cereal rot. Trans. Brit. mycol. Soc., 46:572-576.

Gerdemann, J.W. 1964. The effect of mycorrhiza on the growth of maize. Mycologia, 56:342-349.

Hacskaylo, J., Lilly, V.G. and Barnett, H.L. 1954. Growth of fungi on 3 sources of nitrogen. Mycologia, 46: 691-701.

Hancock, J.G. and Millar, R.L. 1965. Relative importance of polygalacturonate trans-eliminase and other pectolytic enzymes in Southern anthracnose, spring black stem and Stemphylium leaf spot of Alfalfa. Phytopathology, 55:346-355.

Handley, R.W.C. and Sadders, C.J. 1962. The concentration of easily soluble reducing substances in roots and the formation of ectotrophic mycorrhizal association - a re-examination of Björkman's hypothesis. Plant and Soil, 16:42-61.

Hansteen-Cranner, B. 1922. Zur biochemie und physiologie der Grenzschichten Lebender plantzensellen. Meld. Norges Landbrukhögskole, 2:1.

Harley, J.L. 1957. The Biology of Mycorrhiza. Leonard Hill (Books) Ltd. London.

Harley, J. L. and Brierley, J.K. 1954. The uptake of phosphorus by excised mycorrhizal roots of the beech. vi. Active transport of phosphorus from the fungal sheath into the host tissue. New Phytol., 53:240-252.

Harley, J.L. and Brierley, J.K. 1955. The uptake of phosphorus by excised mycorrhizal roots of the beech. vii. Active transport of P^{32} from fungus to host during uptake of phosphate from solution. New Phytol., 54:296-301.

Harley, J.L. and McCready, C.C. 1952. The uptake of phosphorus by excised mycorrhizal roots of the beech (ii) Distribution of phosphorus between host and fungus. New Phytol., 51:56-64.

Harley, J.L. and Waid, J.S. 1955. A method of studying active mycelia on living roots and on other surfaces in soil. Trans. Brit. mycol. Soc., 38(2):104-118.

Hassouna, M.G. and Wareing, P.F. 1965. Possible role of rhizosphere bacteria in the nutrition of Ammophila arenaria. Nature, Lond., 202:467-469.

How, J.E. 1941. The mycorrhizal relations of larch (ii) The role of the larch root in the nutrition of Boletus elegans, Schum. Ann. Bot., Lond., 5:121-131.

How, J.E. 1942. The mycorrhizal relations of larch (iii) Mycorrhizal formation in nature. Ann. Bot., Lond., 6:103-129.

Husain, A. and Dimond, A.E. 1960. Role of cellulolytic enzymes in pathogenesis by Fusarium oxysporum f. lycopersici. Phytopathology, 50:329-334.

Husain, A. and Rich, S. 1958. Extracellular pectic and cellulolytic enzymes of Cladosporium cucumerinum. Phytopathology, 48:316-320.

Johanssen, D.A. 1940. Plant microtechnique. McGraw-Hill Book Co. Inc.

Johansson, M. 1960. A comparison between the cellulolytic activity of white and brown rot fungi. (i) The activity on insoluble cellulose. Physiologia Plant., 19:709-722.

Johnson, L.F., Curl, E.A., Bond, J.H. and Fribourg, H.A. 1960. Methods of studying soil microflora - plant disease relationships. Burgess Publishing Co., Minneapolis.

Katznelson, H., Rouatt, J.W. and Payne, T.M.B. 1954. Observations on the liberation of amino acids by plant roots in relation to desiccation. Nature Lond., 174:1110-1111.

Katznelson, H., Rouatt, J.W. and Payne, T.M.B. 1955. The liberation of amino acids and reducing compounds by plant roots. Plant and Soil, 7:35-48.

Keen, N.T. and Horton, J.C. 1966. The polygalacturonase of Pyrenochaeta terrestris. Phytopathology, 56: 603-609.

Kertesz, Z.I. 1951. The pectic substances. Interscience Publishers, New York.

Kirk, T.K. and Kelman, A. 1965. Lignin degradation as related to the phenoloxidases of selected wood decaying Basidiomycetes. Phytopathology, 55: 739-745.

Kouyeas, V. 1964. An approach to the study of moisture relations of soil fungi. Plant and Soil, 20: 351-363.

Kryuger, L.V. 1961. The dynamics of mycorrhiza formation in meadow grasses. Agrobiologia, 2:226-236.

Lange, J.E. 1936-1940. Flora Agaricina Danica. Vols. 1-5. Copenhagen.

Lange, M. 1952. Species concept in the genus Coprinus. Dansk bot. Ark. 14:20.

Leal, J.A. and Villamueva, J.R. 1962. Lack of pectic enzyme production by non-pathogenic species of Verticillium. Nature, Lond., 195:1328.

Lilly, V.G. and Barnett, H.L. 1951. Physiology of the Fungi. 1st edition. McGraw-Hill Book Co., Inc. N.Y., Toronto, London.

Lindeberg, G. 1944. Uber die physiologie ligninabbauender bodenhymenzeten. Studien an schwedischen Marasmius-Arten. Symb. bot. Upsaliens., 8(2):1-183.

Lindeberg, G. 1946. On the decomposition of lignin and cellulose in litter caused by soil inhabiting hymenomycetes. Ark. Bot., 33A:1-16.

Lindeberg, G. 1948. On the occurrence of polyphenol oxidases in soil inhabiting Basidiomycetes. Physiologia Plant., 1:196-205.

Lochhead, A.G. 1957. Qualitative studies of soil micro-organisms. xv. Capability of the predominant bacterial flora for synthesis of various growth factors. Soil Science, 84:395.

Lundegårdh, H. and Stenlid, G. 1944. On the exudation of nucleotides and flavanone from living roots. Arkiv. Bot. 31(10):1-27.

McArdle, R. 1932. The relation of mycorrhizae to conifer seedlings. J. agric. Res., 44:287-316.

Madelin, M.F. 1956. Studies on the nutrition of Coprinus lagopus Fr. especially as affecting fruiting. Ann. Bot., Lond., 20:307-330.

Madelin, M.F. 1960. Visible changes in the vegetative mycelium of Coprinus lagopus Fr. at the time of fruiting. Trans. Brit. mycol Soc., 43: 105-110.

Mathur, R.S., Barnett, H.L. and Lilly, V.G. 1950. Sporulation of Colletotrichum lindemuthianum in culture. Phytopathology, 40:104-114.

Melin, E. 1922. On the mycorrhizas of Pinus sylvestris L. and Picea abies Karst. J. Ecol., 9:254-257.

Melin, E. 1946. Der einfluss von waldstreuextrakten auf das wachstum von bodenpilzen mit besonderer berücksichtigung der wurzelpilze von bäumen. Symb. bot. Upsalien., 8#r.3.

Melin, E. 1948. Recent advances in the study of tree mycorrhiza. Trans. Brit. mycol. Soc., 30: 92-99.

Melin, E. 1953. Physiology of mycorrhizal relations in plants. Ann. Rev. Plant Physiol., 4:325-346.

Melin, E. 1954. Growth factor requirements of mycorrhizal fungi of forest trees. Svensk bot. Tidskr., 48:86-94.

Melin, E. and Das, V.S.R. 1954. Influence of root metabolites on the growth of tree mycorrhizal fungi. Physiologia Plant., 7:851-854.

Metrod, G. 1941-1942. Les Tricholomes. Revue de Mycologie, N.S.6-7 Supplement pages 22-34.

Moreau, F. 1941-1942. Premiere contribution à l'étude de la microflore des dunes. Revue de Mycologie, N.S.6-7. Supplement 49-94.

Nicolson, T.H. 1959. Mycorrhiza in the Gramineae. i. Vesicular-arbuscular endophytes, with special reference to the external phase. Trans. Brit. mycol. Soc. 42:421-438.

Nicolson, T.H. 1960. Mycorrhiza in the Gramineae. ii. Development in different habitats, particularly sand-dunes. Trans. Brit. mycol. Soc., 43(i): 132-145.

Nobles, M.K. 1958. A rapid test for extracellular oxidase in culture of wood-inhabiting Hymenomycetes. Canad. J. Bot., 36:91-99.

Norkrans, B. 1950. Studies in the growth and cellulolytic enzymes of Tricholoma. Symb. bot. Upsalien., 11:1-126.

Norkrans, B. 1963. Degradation of cellulose. Ann. Rev. Phytopathol., 1:325-350.

Oliver, R.L.A. 1967. A study of factors influencing the distribution of mosses in sand-dune system. M.Sc. Thesis, St. Andrews.

Orton, P.D. 1960. New check list of British Agarics and Boleti. Part 111. Notes on genera and species in the list. Trans. Brit. mycol. Soc., 43(2): 159-439.

Osborne, L.D. and Thrower, L.B. 1964. Thiamine requirement of some wood-rotting fungi and its relation to natural durability of timber. Trans. Brit. mycol. Soc., 47:601-611.

Osvald, H. 1948. Toxic exudates from the roots of Agropyron repens. J. Ecol., 36:193.

Parkinson, D. and Clarke, J.H. 1961. Fungi associating with the seedling roots of Allium porrum L. Plant and Soil, 13:384-390.

Parkinson, D., Taylor, G.S. and Pearson, R. 1963. Studies on fungi in the root region. (1) The development of fungi on young roots. Plant and Soil, 19:332-349.

Pearson, R. and Parkinson, D. 1961. The sites of excretion of ninhydrin positive substances by broad bean seedlings. Plant and Soil, 13:391-396.

Pérombelon, M. and Hadley, G. 1964. Production of pectic enzymes by pathogenic and symbiotic Rhizoctonia strains. New Phytol., 64:144-151.

Pugh, G.J. 1962. Studies on fungi in coastal soils (i) Cercospora salina. Trans. Brit. mycol. Soc., 45:225-260.

Pugh, G.J., Blakeman, J.P., Morgan-Jones, G. and Eggins, H.O.W. 1963. Studies on fungi on coastal soils. Cellulose decomposing species in sand-dune. Trans. Brit. mycol. Soc., 46:565-571.

Rao, A.S. 1959. A comparative study of competitive saprophytic ability in 12 root infecting fungi by an agar plate method. Trans. Brit. mycol. Soc., 42:97-111.

Raper, K.B. and Thom, C. 1949. Manual of the Penicillia. The Williams and Wilkins Co., Baltimore.

Rayner, M.C. 1927. Mycorrhiza: an account of non-pathogenic infection by fungi in vascular plants and cryptogams. New Phytol. Reprint No. 15.

Richards, B.N. 1965. Mycorrhiza development of Loblolly pine seedlings in relation to soil reaction and the supply of nitrates. Plant and Soil, 22: 187-199.

Richards, B.N. and Voigt, G.K. 1964. Role of mycorrhiza in nitrogen fixation. Nature, Lond., 201: 310-311.

Richards, B.N. and Wilson, G.L. 1963. Nutrient supply and mycorrhizal development in Caribbean pine. For. Sci., 9:405-412.

Roboz, E., Barrett, R.W. and Tatum, E.L. 1952. Breakdown of pectic substances by a new enzyme from Neurospora. J. biol. Chem., 195:459.

Romell, L.-G. 1938. A trenching experiment in a spruce forest and its bearing on the problem of mycotrophy. Svensk bot. Tidskr., 32:89.

Romell, L.-G. 1939. The ecological problem of mycotrophy. Ecology, 20:163.

Rouatt, J.W., Peterson, E.H. and Katznelson, H. 1963. Micro-organisms in the root zone in relation to temperature. Canad. J. Microbiol., 9:227-236.

Rovira, A.D. 1956a. Plant root excretions in relation to the rhizosphere effect. i. The nature of root exudate from oats and peas. Plant and Soil, 7:178-194.

Rovira, A.D. 1956b. Plant root excretions in relation to rhizosphere effect. ii. A study of the properties of root exudates and its effect on the growth of micro-organisms isolated from the rhizosphere and control soil. Plant and Soil, 7:195-208.

Rovira, A.D. 1956c. Plant root excretions in relation to rhizosphere effect. iii. The effect of root exudate on the numbers and activity of micro-organisms in soil. Plant and Soil, 7:209-217.

Rovira, A.D. 1965. Interactions between plant roots and soil micro-organisms. Annu. Rev. Microbiol., 19:241-266.

Rovira, A.D. and Harris, J.R. 1961. Plant root excretions in relation to rhizosphere effect. v. Exudation of B-group vitamins. Plant and Soil, 14:199.

Saccardo, P.A. 1888 and 1889. Sylloge Fungorum, Vols. vi and viii. Edwards, J.W. Michigan.

Schroth, M.N. and Hildebrand, D.C. 1964. Influence of plant exudates on root infecting fungi. Ann. Rev. Phytopathol., 2:101-132.

Shirakawa, H.S. 1955. The nutrition of Diplocarpon rosae. Amer. J. Bot., 42:379-384.

Singer, R. 1962. The Agaricales in Modern Taxonomy. 2nd Edit. J. Cramer, New York.

Smart, R.F. 1937. The influence of certain external factors on spore germination in Myxomycetes. Amer. J. Bot., 24:145-159.

Spalding, D.H. 1963. Production of pectinolytic and cellulolytic enzymes by Rhizopus stolonifer. Phytopathology, 53:929-931.

Srivastava, D.N., Echandi, E. and Walker, J.C. 1959. Pectolytic and cellulytic enzymes produced by Rhizopus stolonifer. Phytopathology, 49: 145-148.

Srivastava, D.N. and Walker, J.C. 1959. Mechanisms of infection of sweet potato roots by Rhizopus stolonifer. Phytopathology, 49:400-406.

Stenton, H. 1958. Colonization of roots of Pisum sativum L. by fungi. Trans. Brit. mycol. Soc., 41:74-80.

Strider, D.L. and Winstead, N.N. 1961. Production of cellwall dissolving enzymes by Cladosporium cucumerinum in cucumber tissue and in artificial media. Phytopathology, 51:765-768.

Sulochana, C.B. 1962a. Amino acids in root exudates of cotton. Plant and Soil, 16:312-326.

Sulochana, C.B. 1962b. B-vitamins in root exudates of cotton. Plant and Soil, 16:327-334.

Taylor, G.S. and Parkinson, D. 1961. The growth of saprophytic fungi on root surfaces. Plant and Soil, 15:261-267.

Taylor, G.S. and Parkinson, D. 1964. Studies on fungi in the root region. II. The effect of certain environmental conditions on the development of root surface mycofloras of dwarf bean seedlings. Plant and Soil, 20:34-42.

Taylor, G.S. and Parkinson, D. 1965. Studies on fungi in the root region. iv. Fungi associated with the roots of Phaseolus vulgaris, L. Plant and Soil, 22:1-20.

Thomas, M., Ranson, S.L. and Richardson, J.L. 1956. Plant Physiology, 4th Edition, page 236. J. & A. Churchhill, London.

Vančura, V. 1964. Root exudates of plants. i. Analysis of root exudates of barley and wheat in their initial phase of growth. Plant and Soil, 21:231-248.

Vančura, V. and Hovadik, A. 1965. Root exudates of plants. ii. Composition of root exudates of some vegetables. Plant and Soil, 22:21-32.

Waid, J.S. 1957. Distribution of fungi within decomposing tissue of rye-grass root. Trans. Brit. mycol. Soc., 40:391-406.

Waid, J.S. 1962. Influence of oxygen upon growth and respiratory behaviour of fungi from decomposing rye-grass roots. Trans. Brit. mycol. Soc., 45:479-487.

Wallace, T.J. 1954. The plant ecology of Dawlish Warren. Part ii. The larger fungi. Trans. Devon. Ass. Advmt. Sci., Literature and Art, 86:201-210.

Warcup, J.H. 1951. Effect of partial sterilization by steam or formalin on the fungus flora of an old forest nursery soil. Trans. Brit. mycol. Soc., 34:519-532.

Warcup, J.H. 1959. Studies on basidiomycetes in soil. Trans. Brit. mycol. Soc., 42(1):45-52.

Watling, R. 1963a. Germination of basidiospores and production of fructifications of members of the Agaric family Bolbitiaceae using herbarium material. Nature. Lond., 197:717-718.

Watling R., 1963b. Observations on the Bolbitiaceae. 11. A conspectus of the family. Notes Roy. Bot Gard. Edin., 26(3):289-323.

Webley, D.M., Eastwood, D.J. and Gimingham, C.H. 1952. Development of a soil microflora in relation to plant succession on sand-dunes, including the rhizosphere flora associated with colonizing species. J. Ecol., 40:168-178.

West, P.M. 1939. Excretion of thiamine and biotin by the roots of higher plants. Nature. Lond., 144: 1050-1051.

Willis, A.J. 1963. Braunton Burrows: The effects on the vegetation of the addition of mineral nutrients to dune soils. J. Ecol., 51:353-374.

Willis, A.J. 1965. The influence of mineral nutrients on the growth of Ammophila arenaria. J. Ecol., 53: 735-745.

Willis, A.J. and Yemm, E.W. 1961. Braunton Burrows: Mineral nutrient status of the dune soils. J. Ecol., 49:377-390.

Winstead, N.N. and McCombs, C.L. 1961. Pectinolytic and cellulolytic enzyme production by Pythium aphanidermatum. Phytopathology, 51:270-273.

Winstead, N.N. and McCombs, C.L. 1963. Production of pectinolytic and cellulolytic enzymes by cucurbit anthracnose fungi. Phytopathology, 53:961-964.

Zak, B. 1964. Role of mycorrhizae in root disease. Ann. Rev. Phytopathol., 2:377-392.